University of Alberta

Growth Hormone in Chick Embryogenesis

by

Christopher Derek Martin Johnson

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Physiology

Edmonton, Alberta

Spring 1998

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ABSTRACT

Growth hormone is the major regulator of postnatal growth, but its role in the growth and development of the embryo or foetus is uncertain. Indeed, chicken embryos continue to grow after decapitation and the removal of the pituitary gland. GH-secreting cells of the anterior pituitary gland are not functional until mid-late gestation and it is therefore likely that pituitary GH is not involved in embryonic growth. However, GH is now known to be produced by extrapituitary tissues, and it is possible that embryonic growth and development is dependent upon this GH source.

Immunoreactive GH-like proteins were detected in extra-pituitary tissues of the developing chick embryo, prior to the functional development of the GH-secreting cells of the anterior pituitary gland. GH-immunoreactive cells were found to be ubiquitously present in the ED 3 embryo. In later ontogenic stages, GH-immunoreactive cells were found in the Wolffian duct, mesoderm, amnion, and cartilage cells. In addition growth hormone receptor (GHR) was shown to be present in several embryonic tissues.

GH-like proteins were found to be present within neural tissues of the developing embryo. GH-immunoreactivity was found in the neural tube and developing brain (cerebral cortex, cerebellum, choroid plexus, and subtrochlear organ) optic and otic vesicles, nerve ganglia, and in the developing eye. GHR-immunoreactivity was also detected in neural tissues.

These results challenge the widely held view that GH has no role during embryonic growth and development, and the presence of GH and GHR in the same tissues raises the possibility that GH may have autocrine or paracrine roles in the regulation of embryonic growth and development.

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DEDICATIONS

My parents were fundamental in my destiny and they are among the most impressive educators I have seen. Without their love, support and advice I'm sure I would not be where I am today. They always supported my aspirations and sacrificed a great deal so that I could continue my studies. I therefore, dedicate this thesis foremost to them:

Caroline Mary and Derek Thomas

I also remember my sisters Helen Clare and Katherine Teresa who are both attending university in England. I wish you the best of luck in all your endeavours. I have missed a large part of my brother Joseph Anthony growing up and I hope we will become best friends in the future.

There have been many people who have had a large impact on my life. One such person is my aunt Caroline Christina. All who know this remarkable lady will agree that her exuberance for life, unconditional generosity and amazing sense of humour, are a welcome break from this often depressing world.

For Elena Antoaneta Nedea and Roberto Matteucci.

ACKNOWLEDGMENTS

I would like to thank Dr. Stephen Harvey for his support and guidance regarding this project. He allowed me considerable freedom in his laboratory and he really went out of his way to welcome me to Canada. I am particularly grateful with his exceedingly close attention to my thesis. He is an excellent writer and I hope I have improved my own skills through his corrections.

I also thank Dr. Kerry Hull for her careful attention to laboratory and technical problems that I faced from time to time. Kerry was always needed around the lab and was surely missed when she left. Very many thanks to Dr. Esmond Sanders for his input and support. Thanks also to Dr. Greer. Special thanks to Dr. Foxcroft for being the external at such short notice. I would like to acknowledge Dr. Dusyk for his help regarding computer problems. He always had a little time to spare and is invaluable to the department. Thanks to Dr. Michael Wride for his scientific advice and to Dr. Keith Bagnall for heap with chick anatomy.

There are many people to thank for their friendship and support during my time at the University of Alberta. I particularly thank those people who allowed me to be "who" I am. I thank Dr. Michael Wride and Dr. Catherine Wride for their staunch friendship and advice. I'll always remember the laughs over fine bone china!!! I remember Karen Thethi for her friendship and for those fun times we had. I would like to thank many others whom I also regard very highly: Sue Hopkirk, Colleen Norman, Sarah and Amy Piggott, Bill Keyes, Mark Ridyard, Christophe Mattonat, Alessandra, Christophe de Cassan, Kamran Fathimani, Poonit Sharma, Debbie O'Neill, Dr. Tony Ho, Dr. Connie Chik, Dr. Christopher Cheeseman, Dr. David Olson, Dr. Suzanne

Barton, Andrew Hirsh, Victoria Inglis, Nicholas Malone, Helle Svaerke, Sigrun Vala Bjornsdottir and so many others. Last but not least a big thankyou to my parents for their financial support.

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LIST OF ABBREVIATIONS

APAAP	alkaline phosphatase, anti-alkaline phosphatase
BBB	blood-brain-barrier
bGH	bovine growth hormone
bp	base pairs
cDNA	complementary deoxyribonucleic acid
cGH	chicken growth hormone
CNS	central nervous system
CSF	cerebrospinal fluid
DAB	diaminobenzidine
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
hGH	human growth hormone
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFs	insulin-like growth factors
kb	kilobase
kDa	kilodalton
LH	luteinizing hormone
mRNA	messenger ribonucleic acid

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIT-1/GHF-1	pituitary transcription factor-1/growth hormone factor-1
PRL	prolactin
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SRIF	somatostatin
T_3	L-3,5,3-triiodothyronine
TBS	tris buffered saline
tGH	turkey growth hormone
TRH	thyrotrophin releasing hormone
TSH	thyroid stimulating hormone

Chapter One

Literature Review:

Growth Hormone Expression During Chick Embryogenesis

I. GENERAL INTRODUCTION

For almost one hundred years, the necessity of the pituitary gland for postnatal growth has been recognized. In the 1920's the ability of crude pituitary extracts to restore growth in hypophysectomised animals was first demonstrated, and the protein responsible, growth hormone (GH), was subsequently purified from bovine (Li and Evans, 1944) and human (Li and Papkoff, 1956) pituitary glands.

GH is the main biological mediator of postnatal growth and it is well established that a deficiency of this pituitary hormone can result in dwarfism whereas an excess can result in gigantism. GH induces postnatal growth in a classical *endocrine* fashion. It is secreted from the pituitary gland into the bloodstream and induces the cellular proliferation of most tissues, either directly, or indirectly through the induction of insulin-like growth factors (IGF's).

Paradoxically, embryonic/foetal growth has long been considered to be GH-independent and directly regulated by local growth factors or placental hormones. The ontogenic appearance of pituitary GH-secreting cells and the onset of pituitary GH synthesis and secretion occurs during mid-late embryogenesis and early embryonic/foetal growth is therefore independent of pituitary GH. The hypothesis of this thesis is, however, that early embryonic growth may be dependent upon the action of GH produced in extra-pituitary tissues.

Until recently GH was thought to be solely produced within the pituitary gland; however, recent work has clearly demonstrated the extrapituitary production of

GH in a wide variety of adult animal tissues that are target sites for GH action (Harvey and Hull, 1997). It is possible that this locally produced GH can exert biological effects via autocrine or paracrine modes of action. The ontogeny and distribution of extra-pituitary GH-secreting cells in the chick embryo was therefore the focus of this study.

II. AVIAN PITUITARY GH

A. GH Chemistry

GH has been purified from the pituitary glands of chickens (Harvey and Scanes, 1977), turkeys (Farmer *et al.* 1974), ducks (Papkoff and Hayashida, 1974), and ostriches (Papkoff *et al.* 1982). In the chicken, pituitary GH is mostly present as a monomer with an estimated molecular weight of between 22 - 27 kDa (Harvey and Scanes, 1977; Lai *et al.* 1984; Leung *et al.* 1984; Souza *et al.* 1984; Houston and Goddard, 1988; Aramburo *et al.* 1989, 1990, 1991; Render *et al.* 1995b). Other GH moieties of 16 kDa have also been reported (Aramburo *et al.* 1989; Render *et al.* 1995b) and possibly reflect proteolytic processing. Dimeric (40-52 kDa) forms of the protein also exist (Aramburo *et al.* 1989) and these may result from disulphide bonding (Houston and Goddard, 1988). Some moieties may also be glycosylated (Berghman *et al.* 1987) or phosphorylated (Aramburo *et al.* 1990; Aramburo *et al.* 1992).

B. GH Gene

The chicken GH (cGH) gene has been cloned and its nucleotide sequence has been determined (Tanaka *et al.* 1992). The gene consists of five exons and four introns, as in the mammalian GH gene, although it is larger due to its greater intron size. The promoter region contains a short (24bp) sequence which is highly homologous to the antisense sequence for the PIT-1 transcription factor in the rat GH gene (Tanaka *et al.* 1992). As the pituitary GH gene is thought to be specifically activated by PIT-1 in the pituitary gland (Simmons *et al.* 1990), the presence of such a sequence in the cGH gene strongly suggests that the PIT-1 transcription factor also regulates the expression of the GH gene in the chicken pituitary.

C. GH-Secreting Cells

1. Distribution

GH is produced by somatotrophs within the anterior pituitary gland. In mammals, somatotrophs tend to be widely distributed within the gland, whereas in lower vertebrates they are often localized to specific regions. In birds, 90% of pituitary GH-secreting cells are confined to the caudal lobe of the adenohypophysis, the rest being located toward the ventral end of the cephalic lobe (Figure 1.1) (Jozsa *et al.* 1979; Mikami, 1986; Thommes *et al.* 1987; Berghman *et al.* 1992; Malamed *et al.* 1993; Hull *et al.* 1992; Lopez *et al.* 1995). The abundance of GH-secreting cells in the cephalic lobe appears to be more labile than in the caudal lobe and in adult

females is related to reproductive status (Ramesh *et al.* 1995). GH mRNA expression closely matches somatotroph distribution in the caudal lobe (Kansaku *et al.* 1994; Kansaku *et al.* 1995; Reiprich *et al.* 1995), although islands of GH mRNA expressing cells have been located within the cephalic lobe (Kansaku *et al.* 1995; Kansaku *et al.* 1995; Hull *et al.* 1997).

2. Morphology

In chickens, as in all species, somatotrophs have a characteristic morphology (Tai and Chadwick, 1977; Malamed *et al.* 1985; Malamed *et al.* 1988; Malamed *et al.* 1993). They are round, oval or triangular in shape, with well developed Golgi and granular endoplasmic reticulum. They have a large nucleus with heterochromatin, prominent nucleoli, numerous nuclear pores, and are packed with dense-core secretory vesicles. Mitochondria are oval or elongated with oblique cristae.

GH is stored within secretory granules in somatotrophs. These membrane-bound vesicles are typically oval, large (260 nm to 400 nm in diameter) and electron dense. In chickens, pituitary somatotroph number, granule size and content increase with age in adults (Malamed *et al.* 1985; Malamed *et al.* 1988; Vasilatos-Younken *et al.* 1988; Malamed *et al.* 1993; Porter *et al.* 1995a).

3. Heterogeneity

In mammals it is well established that somatotrophs exist as heterogeneous sub-populations according to secretory granule size and content, spatial distribution, functionality, and the rate of degranulation (Snyder *et al.* 1977; Ohlsson *et al.* 1988; Thorpe and Wallis, 1991; Mohanty *et al.* 1993; Torronteras *et al.* 1993). In chickens, somatotrophs are spatially heterogeneous and exist as either cords or clumps (containing 4-15 cells) or as isolated cells (Hull *et al.* 1997) (Fig 1.1). Furthermore, these cells differ in their immunoreactivity with GH antibodies. Cells located to the periphery of the caudal lobe show denser staining than those located towards the centre. Cells in the cephalic lobe are also less densely stained than those from the caudal lobe (Hull *et al.* 1997). This is also seen in the turkey pituitary gland (Ramesh *et al.* 1996) and may reflect differences in the amount of GH stored within different GH-secreting cells.

The heterogeneity in pituitary GH staining could, however, also be due to the presence of different GH molecular variants or isoforms (Houston and Goddard, 1988) or be indicative of functional differences in somatotroph sub-populations. For instance, somatotrophs in the cephalic lobe of the chicken pituitary gland (unlike those in the caudal lobe), are not responsive to the stimulatory effects of GHRH on GH release and gene expression (Kansaku *et al.* 1994). Functionally heterogeneous somatotrophs are also present within the mammalian pituitary gland (Perez and Hymer, 1990).

4. Differentiation

The pattern of anterior pituitary cell differentiation has been extensively studied in mammals (Voss and Rosenfeld, 1992; Anderson and Rosenfeld, 1994). The first cells to appear are the adrenocorticotropin (ACTH)-secreting cells, which appear to differentiate from the anterior pituitary anlagen, without extra-pituitary stimulation. The second cell type to appear is the gonadotroph, followed by the thyrotrophs, somatotrophs and lactotrophs, respectively. The precursor cells for each of these remains unclear. A transcription factor, PIT-1, is known to be essential for the expression of GH, thyrotrophin-stimulating hormone (TSH) and prolactin (PRL) in mammalian pituitaries (Voss and Rosenfeld, 1992; Anderson and Rosenfeld, 1994). In contrast, the differentiation of pituitary cells in birds is largely unknown (Porter, 1997). The timing of the differentiation of each type of cells is unclear, particularly for somatotrophs, with reports of the GH-secreting cells first appearing as early as 4.5 days (Thommes *et al.* 1987) of the 21 day incubation period or as late as 16 days (Porter *et al.* 1995a).

5. Regulation

The regulation of GH secretion in birds is similar to that in mammals in that it is multifactorial and dynamic during ontogeny and according to physiological status (see Harvey *et al.* 1991; Harvey, 1993 for detailed reviews). GH release by somatotrophs is primarily controlled by the hypothalamus through the interaction of

thyrotropin-releasing hormone (TRH) that stimulates, and somatostatin (SRIF) that inhibits, GH secretion. Although a GH-releasing hormone (GHRH) has been identified in the chicken hypothalamus (McRory *et al.* 1997), its participation in the regulation of GH secretion in birds is uncertain (Harvey, 1998). In the absence of hypothalamic tissues, very little GH synthesis or release occurs *in vitro* (Harvey *et al.* 1991), although the persistence of both indicates some autonomous control.

D. Mechanism of GH Action

1. Introduction

Growth hormone has a broad range of actions on growth, differentiation, and intermediary metabolism. The biological actions of GH are initiated by its binding to specific cell-surface receptors, which are present in most tissues. This ligand activation of the receptor leads to the induction of numerous intracellular signal-transduction cascades and to altered expression of specific genes in target cells (Carter-Su *et al.* 1996; Burnside *et al.* 1997 for reviews). The proteins encoded by some of these genes (eg. insulin-like growth factors (IGF's)) act as intermediaries for GH in growth and metabolism.

2. GH Receptor

The growth hormone receptor (GHR) is a member of the cytokine receptor superfamily (which also contains the prolactin receptor), and contains a number of

conserved features. They are single transmembrane-spanning proteins, with an extracellular ligand-binding domain, a transmembrane domain and intracellular domain, which is involved in signal transduction (Burnside *et al.* 1997).

The rabbit GHR gene was the first GHR gene to be cloned (Leung *et al.* 1987) and since then structural information on the GHR from many other species, including human, cow, sheep, rat, mouse and chicken (reviewed in Kelly *et al.* 1991; Burnside *et al.* 1997), has been obtained. The predicted chicken GHR shares about 50% homology with the deduced human GHR in amino acid sequences.

3. GH Binding and Receptor Dimerization

Growth hormone has two binding sites in its tertiary structure (Cunningham *et al.* 1991). These sites are each thought to interact with one GHR and therefore, one GH molecule is hypothesized to bind to two GHRs (Colosi *et al.* 1993). This binding results in receptor dimerization, which in turn induces the activation of JAK kinases and MAP kinases, tyrosol phosphorylation of insulin receptor substrate 1 and 2 (IRS-1, -2), an increase in intracellular Ca^{2}+, and activation of the latent transcriptional factors Stats 1, 3, and 5 (Carter-Su *et al.* 1996). These events, in turn, can initiate signalling pathways involving other tyrosine kinases or phosphatases.

GH rapidly induces the expression of early-response genes, including *c-fos* and *c-jun* (Doglio *et al.* 1986; Gurland *et al.* 1990; Slootweg *et al.* 1991). These encode for transcription factors implicated in cell growth and differentiation. These

factors may, in turn, regulate gene expression involved in long-term responses to GH. Other GH responsive genes include members of the cytochrome P450 family, lipoprotein lipase, insulin, and members of the serine protease inhibitor (Spi) family (see Burnside *et al.* 1997 for review). IGF-1 gene expression is also stimulated by GH in the liver and possibly other tissues, by predominantly transcriptional mechanisms (Bichell *et al.* 1992).

E. Pituitary GH and Growth

1. Introduction

Growth can be defined as a phenomenon which is characterized by hyperplasia (increase in the number of differentiated cells) and subsequent hypertrophy (increase in the size of cells), and therefore, involves a developmental increase in mass, including an increase in length and total body weight of an organism. Growth results in the differential growth of tissues and organs and the functional integration of organ systems. This phenomenon is influenced by a multitude of factors including hereditary, maternal and foetal nutrition and the developing endocrine system.

2. Mammalian Postnatal Growth

The role of GH role in mammalian growth is well documented (reviewed Lindahl *et al.* 1991). GH is secreted from the pituitary gland, and travels to sites of

action via the bloodstream. At hepatic sites, GH binds to the GHR and induces the synthesis of IGF-I, which acts as an intermediary for GH and promotes the proliferation and differentiation of growth plate chondrocytes (Lindahl *et al.* 1991). GH can also induce the expression of IGF-I directly within the growth plate (Isaksson *et al.* 1987). Furthermore, GH can also act directly upon chondrocytes (Ohlsson *et al.* 1992a,b) by binding to its own receptor on the cell surface (Bentham *et al.* 1993) and augmenting cell proliferation and differentiation independently of IGF-I (Isaksson *et al.* 1982; Schlechter *et al.* 1986; Isaksson *et al.* 1987).

3. Chicken Postnatal Growth

The role of GH in avian postnatal growth in general, and in longitudinal bone growth in particular, is far from clear. GH is required for postnatal growth in chickens, as hypophysectomy (Huybrechts *et al.* 1985; Scanes *et al.* 1986; Lazarus *et al.* 1988; Vanderpooten *et al.* 1991; Morishita *et al.* 1993) and administration of GH antisera (Scanes *et al.* 1977) reduces skeletal growth (but does not abolish it). However, GH replacement therapy partially restores these affects (Scanes, 1997). The effects of exogenous pituitary GH administration in pituitary-intact birds is, however, inconsistent and uncertain. For instance, the daily injection of purified cGH to 4-week-old cockerels produced a transient increase in growth rate in one study (Leung *et al.* 1986), whereas, in others, administration of GH to young chickens did not alter growth performance (Scanes *et al.* 1984; Burke *et al.* 1987; Cogburn *et al.* 1989) or

longitudinal bone growth (Burke *et al.* 1987; Cravenor *et al.* 1989). Moreover, genetic strains of birds with fast growth rates exhibit the lowest concentrations of circulating GH (Goddard *et al.* 1988).

It is probable that GH acts via IGF-I in chicken postnatal growth, as GH appears to increase circulating levels of IGF-I in hypophysectomised and intact birds (Leung *et al.* 1986). The rise in IGF-I levels during postnatal development also occurs subsequent to a rise in plasma GH concentrations (Harvey *et al.* 1979; Huybrechts *et al.* 1985), suggesting a negative feedback effect of IGF-I on GH secretion.

In contrast to mammals, however, no increase in cell proliferation was observed after treatment of avian growth plate chondrocytes with cGH (Rosselot *et al.* 1992, 1994; Monsonego *et al.* 1995), even though the GHR is expressed on these cells (Monsonego *et al.* 1993) and is capable of binding chicken and human (h)GH in culture (Monsonego *et al.* 1993). Although GH by itself did not augment cell proliferation, changes in gene expression, synthesis and activity of chondrocyte differentiation markers were observed in these studies (Monsonego *et al.* 1995). cGH caused an increase in collagen type II gene expression (a marker for proliferation of chondrocytes) and sustained the proliferative state and inhibited their differentiation into hypertrophic cells. These cells are, therefore, susceptible to growth factors for a longer period of time during bone formation.

Insulin-like growth factor-I gene expression occurs in the chicken liver and other tissues throughout postnatal growth (Kikuchi *et al.* 1991; Rosselot *et al.* 1995; Tanaka *et al.* 1996). Again the data pertaining to IGF-I and postnatal growth is, however, unclear. Comparison of plasma IGF-I concentrations in birds genetically selected for high growth rates, are higher than those with slower growth rates (Scanes *et al.* 1989), although the opposite situation has also been reported (Huybrechts *et al.* 1985). Moreover, IGF-I gene expression has been shown to be independent of GH in GH-resistant strains of poultry (Tanaka *et al.* 1996), and exogenous GH failed to induce IGF-I production in chick chondrocytes (Halevy *et al.* 1996).

III. EXTRA-PITUITARY GH

A. Introduction

It is now known that GH and GHRs are expressed in a wide variety of tissues in many species (Table 1.1). The production of GH within these sites is likely to act locally, since circulating GH is derived exclusively from pituitary GH and is undetectable following hypophysectomy or pituitary ablation (McGuinness and Cogburn, 1990). Furthermore, in many of these sites, GH has been shown to have biological activity unrelated to growth.

B. GH: A Neural Paracrine/Autocrine

The brain is an extra-pituitary site of GH synthesis (see Harvey *et al.* 1993 for review). The GH gene is expressed in the rat telencephalon, diencephalon, mid-brain and metencephalon (Gossard *et al.* 1987; Martinoli *et al.* 1991) and in the chicken the cDNA of the neural GH gene is homologous to pituitary GH cDNA (Render *et al.* 1995). GH moieties of smaller and larger size have been observed suggesting a number of GH like proteins may be expressed in the brain (Noteborn *et al.* 1993; Lechan *et al.* 1981; Lechan *et al.* 1983), although Render *et al.* (1995) only found the monomeric moiety in the chicken brain.

Neural GH is possibly an evolutionary precursor to pituitary GH as GH-immunoreactivity is found in foetal rat brains before it appears in the pituitary (Hojvat *et al.* 1982b). It also occurs in the neural tissues of primitive vertebrates (Wright, 1986) and in invertebrates that lack pituitary glands (Swinnen *et al.* 1990; Werkman *et al.* 1991; Toullec *et al.* 1992).

Neural GH may act in autocrine or paracrine ways, as GH-binding sites and GHR mRNA are found within the brains of vertebrates from fish (Sanchez *et al.* 1991), birds (Fraser *et al.* 1990) and mammals (Lopez-Fernandez *et al.* 1996). Furthermore, the regions of GHR mRNA expression overlap those of GH gene expression (Harvey *et al.* 1993). Indeed, it is now recognized that the brain is a GH target tissue (Harvey *et al.* 1993; Han, 1995; Nyberg and Burman, 1996). Neuronal and glial growth and differentiation have been shown to be GH-dependent and severe

deficits occur in GH-deficient states (Noguchi, 1996). GH has also been shown to exhibit neuromodulatory pathways (Burman *et al.* 1996; McGauley *et al.* 1996) and to possess regulatory roles in motor activity, breathing, learning, memory, sleep, feeding and other central behaviours (Sartorio *et al.* 1996; McGauley *et al.* 1996). These actions may be induced by GH produced within the brain. For instance, the occurrence of GH in the ventromedial nucleus (VMN) of the hypothalamus, may be responsible for the stimulatory effect of GH on feeding (Hojvat *et al.* 1986).

C. GH: An Immune Paracrine/Autocrine

There is abundant evidence to show the production of GH by immune cells and the autocrine and paracrine actions of this locally produced GH (reviewed by Weigent, 1996).

Several immune cells have been shown to express the GH gene. Rat and human lymphocytes secrete GH-like proteins in vitro (Weigent *et al.* 1988; Varma *et al.* 1993; Hattori *et al.* 1994), as do leukocytes in vivo (Baxter *et al.* 1991). GH mRNA is also present in human and rat peripheral blood leukocytes (Weigent *et al.* 1988) and in cells of the spleen, thymus and bone marrow. In chickens, GH immunoreactivity is found in the spleen, thymus and bursa of Fabricius (Render *et al.* 1995a).

It is well established that immune cells are target sites for GH action. GH-immunoreactivity is present on the membrane surfaces of circulating immune cells

and immortalised lymphocyte cell lines (Badolato *et al.* 1994; Rapaport *et al.* 1995). In mammals, these receptors are present in sub-populations of B- and T- lymphocytes and natural killer cells (NKC) and particularly in B- cells (Badolato *et al.* 1994). In birds, the GHR is largely associated with macrophages and other large mononuclear non-lymphoid cells (Hull *et al.* 1996b). The GHR gene is also similarly expressed in the thymus and spleen of rats (Mertani *et al.* 1995) and in the bursa of Fabricius of birds (Hull *et al.* 1996) and the head kidney of fish (Calduch-Giner *et al.* 1995). Furthermore, ^{125}I-labelled GH membrane binding sites are present on lymphocytes (Kover *et al.* 1984) and thymocytes (Ban *et al.* 1991).

GH plays an integral role in the maintenance and functioning of the immune system. Pituitary GH deficiency results in impaired immune function (Nagy and Berczi, 1978; Khansari and Gustad, 1991; Corpas *et al.* 1993) and this can be restored by GH therapy (Murphy *et al.* 1992b). Indeed, exogenous GH can increase thymic size, thymocyte proliferation/differentiation, the secretion of thymulin, the activation and proliferation of lymphocytes, increased production of cytokines, and the activation of monocytes, macrophages, phagocytosis, the generation of superoxide anions and the intravascular migration of immune cells (Aurernhammer and Strasburger, 1995).

In addition to pituitary GH, it has been shown that immune GH is physiologically important in the induction of leukocyte function. The blockade of local GH synthesis in rat lymphocytes, by the addition of a GH antisense

oligonucleotide, results in a reduction of cellular proliferation (Weigent *et al.* 1991). Furthermore, the autocrine actions of GH within leukocytes have also been indicated by their suppression of IGF-I production when GH action is blocked by GH antibodies (Baxter *et al.* 1991). The immunoneutralization of endogenous GH similarly blocks the proliferation of rat thymic epithelial cells (Sabarwal and Varma, 1996) and T-lymphocyte proliferation (Lattuada *et al.* 1996).

D. GH: A Placental Paracrine/Autocrine

Pituitary GH is expressed in humans by a gene (hGH-N) which is a member of the GH-chorionic somatomammotropin (hCS) family. One member of the family, the hGH-V gene, is expressed by syncytiotrophoblasts and epithelial cells of the placenta (Cooke and Liebhaber, 1997). It appears that the hGH-V gene is only transcribed in the placenta, and its expression is restricted in other tissues by inhibitory proteins.

Placental GH is distinct from pituitary GH in that it differs in 13 of its 191 amino acid sequence (Lacroix *et al.* 1996). This protein is also much larger (33 kDa) as a result of extensive glycosylation at a unique N-linked glycosylation site. This glycosylation also makes the protein more basic than pituitary GH. A placental GH (mGH-V) is also expressed in the rhesus monkey (Golos *et al.* 1993) and the placenta of rodents expresses a novel family of GH-like proteins (Ogilvie *et al.* 1990).

Placental GH has extensive mitogenic activity (Nickel *et al.* 1990; Macleod *et al.* 1991), and it has been shown to enhance placental IGF-I production (Challier *et al.* 1991) and to stimulate endometrial cell growth (Strowitzki *et al.* 1991). It is possible therefore, that placental GH could alter placental growth and differentiation in paracrine or autocrine modes of action, since the GHR is present in the placenta (Frankenne *et al.* 1988; Hill *et al.* 1992; Urbanek *et al.* 1993) from at least 10-12 weeks of gestation in humans (Hill *et al.* 1992).

E. GH: A Mammary Gland Paracrine/Autocrine

Growth hormone is produced by the dog (Selman *et al.* 1994), cat (Mol *et al.* 1995), and human mammary gland (Mol *et al.* 1995). The synthesis of mammary GH in felines and canines is inducible by progestins and is correlataed with the hyperplasia and proliferation of mammary gland cells (Mol *et al.* 1995). GH also has been shown to have growth-promoting effects on human mammary cancer cells in vitro (Benlot *et al.* 1997). It is thought that GH and IGF-I induce the growth and differentiation of mammary tissues through paracrine interactions (Mol *et al.* 1996).

F. GH: A Gonadal Paracrine/Autocrine

GH-immunoreactive proteins are present in the reproductive tract of embryonic day (ED) 18 foetal mice (Nguyen *et al.* 1996) and GH mRNA has been detected in the human testis (Untergasser *et al.* 1996).

The GHR is expressed in the foetal male and female rat reproductive system. In males, it is present in the Wolffian duct, ureter, epididymis, vas deferens, seminal vesicles and gonads (Garcia-Aragon *et al.* 1992), whereas in females it is found in the oviduct and uterine lining, in granulosa and thecal cells and in oocytes (Lobie *et al.* 1990).

The possibility that gonadal GH possesses autocrine/paracrine actions is strongly suggested by the ability of GH antiserum to block Wolffian duct differentiation in vitro in mice (Nguyen *et al.* 1996). Exogenous GH also stabilizes the Wolffian duct in female foetuses in vitro (Nguyen *et al.* 1996).

G. GH: A Growth/Differentiation Factor?

There is now considerable evidence that pituitary GH or systemically administered GH can promote the growth of discrete organs and tissues in an endocrine way (Scanes and Daughaday, 1995). It is also possible that extra-pituitary GH may induce cellular proliferation and differentiation through autocrine/paracrine mechanisms, similar to the local induction of growth by IGFs and other growth factors. In addition to inducing growth, GH may also act as a differentiation factor. Indeed, the differentiation of the pituitary (Flint *et al.* 1992), mammary gland (Weigent *et al.* 1991a), male internal genitalia (Nguyen *et al.* 1996) and lymphoid elements (Hooghe *et al.* 1993) have been shown to be GH-dependent. Several fibroblast lines (3T3-F442A, 3T3-L1, Ob 1771) are similarly induced to differentiate

into adipocytes by GH in vitro (Catalioto *et al.* 1992). Furthermore, GH mRNA has been detected in human skin fibroblasts (Palmetshofer *et al.* 1995), in which GHRs and GHR mRNA are present (Oakes *et al.* 1992), and it is therefore possible that locally produced GH may augment fibroblast differentiation. The differentiation of prechondrocytes into chondrocytes is similarly induced by GH (Loveridge and Farquharson, 1993).

There is also considerable evidence to suggest that GH is a paracrine growth and differentiation factor in the haemopoietic system (reveiwed in Hooghe *et al.* 1993), acting directly or indirectly via IGF-I. GH and GH mRNA are present within haemopoietic tissues (Gala, 1991), in which GHRs are found (Matera *et al.* 1988). Moreover GH binding to its receptors induces proliferation (Berczi *et al.* 1991) or differentiation (Gala, 1991) of mammalian leukocytes.

H. Regulation of Extra-Pitutary GH

In most species, the regulation of pituitary GH is dependent upon hypothalamic releasing factors that stimulate (growth hormone-releasing hormone, GHRH) or inhibit (SRIF) GH release. It is also possible that the regulation of extra-pituitary GH could be regulated by similar factors in paracrine or autocrine ways.

A "mini hypophysis" is, for instance, present within immune tissues. Growth hormone-releasing hormone and GHRH mRNA are found in rat and human lymphocytes (Stephanou *et al.* 1991; Weignet *et al.* 1991) and in rat spleen

(Matsubara *et al.* 1995). GHRH receptors are found in rat thymocytes and splenocytes (Guarcello *et al.* 1991; Matsubara *et al.* 1995). It seems that GHRH affects GH synthesis in rat leukocytes by autocrine or intracrine mechanisms, because GHRH antisense oligonucleotides (but not GHRH antibodies) block GHRH-induced leukocyte GH synthesis (Guarcello *et al.* 1991).

Somatostatin mRNA has been detected in rat lymphocytes (Aguila *et al.* 1996) and in the Bursa of Fabricius of chickens (Aguila *et al.* 1996). The role of SRIF in regulating immune GH function is, however, uncertain. Nevertheless, inhibitory effects of SRIF on macrophage and immune function have been demonstrated, presumably as a result of GH suppression (Aguila *et al.* 1996; Chao *et al.* 1995).

Growth hormone-releasing hormone mRNA and protein are also present in human (Berry *et al.* 1992), mouse (Endo *et al.* 1994; Yamaguchi *et al.* 1995), and rat (Matsubara *et al.* 1995) placentae, indicating that local GHRH may have actions. GHRH receptors have similarly been detected in the rat placenta (O'Carroll *et al.* 1994). However, the very sensitive RT-PCR/Southern blotting technique was unable to detect GHRH receptor mRNA (Matsubara *et al.* 1995) and placental GH production has not been shown to respond to exogenous GHRH (de Zegher *et al.* 1990). It therefore, remains unclear whether a "mini hypophysis" is present within the placenta.

Growth hormone-releasing hormone mRNA has similarly been found in the human (Pescovitz *et al.* 1990; Berry *et al.* 1992) and rat testis (Tsagarakis *et al.*

1991; Matsubara *et al.* 1995), and in the human and rat ovary (Matsubara *et al.* 1995). SRIF is also present in the rat testis (Pekary *et al.* 1984). The functions of these regulatory peptides in these reproductive tissues are unknown, although GHRH receptors have been detected in Sertoli cells (Srivastava *et al.* 1994).

A "mini-hypophysis" may also exist in the pituitary gland, as GHRH and SRIF (peptides and receptors) are synthesised within the gland (Matsubara *et al.* 1995; O'Carroll *et al.* 1994). It is therefore possible that these locally produced peptides could modulate GH synthesis and secretion. The synthesis and release of GH within the brain may also be regulated by locally produced GHRH and SRIF (peptide and receptor), since they are all colocalised within hypothalamic nuclei (Matsubara *et al.* 1995; O'Carroll *et al.* 1993).

IV. EMBRYONIC/FOETAL GROWTH

A. Introduction

Embryonic/foetal growth is a dynamic process that begins at fertilization and terminates at hatching or birth. As in postnatal growth, cellular hyperplasia, differentiation and hypertrophy are the characteristics of this phenomenon. The regulation of embryonic/foetal growth is poorly understood and this is further complicated by the physiological differences between animal models (i.e. placental versus non-placental animals).

The factors involved in embryonic/foetal growth may be of maternal origin or originate from the embryo or foetus. The embryonic or foetal endocrine system may be a source of such factors.

1. Embryonic Endocrine System

Bouin and Ancel (1903) were the first researchers to postulate that hormones may act as growth factors during definite time periods throughout embryonic/foetal growth and development, after their observation of "active-looking" interstitial cells in the foetal pig testis before the differentiation of the genital tract. They suggested that the internal secretions of the foetal testes may regulate the development of male sex characteristics. Further support for functional foetal endocrine glands was provided by the finding that the thyroid gland was responsible for inducing growth and metamorphosis in anuran tadpoles (reviewed in Thommes and Woods, 1993).

Despite these early studies the role of the embryonic/foetal endocrine system in the regulation of growth and development is still poorly understood. There is evidence to suggest a role for hormones in the growth, development and differentiation of the avian embryo or mammalian foetus (reviewed in Thommes and Woods, 1993; Evain-Brion, 1994) These hormones may be developmentally regulated, appearing at certain ages or stages of development. Furthermore, hormones involved in embryonic/foetal growth may possess biological activity through autocrine/paracrine modes of action like many of the peptide growth factors. For

instance, luteinizing hormone (LH), which is primarily involved in regulating reproductive function in adults, was recently shown to be expressed in the lung bud epithelia and stomach of the early chicken embryo (Shirasawa *et al.* 1996). This may indicate that many pituitary hormones possess hitherto unsuspected roles in embryogenesis.

B. Embryonic Growth is Independent of Pituitary GH

1. Introduction

Although it is well established that postnatal growth and development are dependent upon the endocrine actions of pituitary GH it has long been thought that embryonic/foetal growth and development are independent of these actions. Indeed, embryonic and foetal growth occurs before the pituitary has commenced production and secretion of GH (Harvey and Scanes, 1977; Hemming *et al.* 1986; Malamed *et al.* 1993; Porter *et al.* 1995a; Porter *et al.* 1995b), during which time it was thought that growth is predominantly regulated by growth factors and placental hormones (reviewed by Geffner, 1996). However, GH has been shown to have actions in the chick embryo (Table 1.4). Consequently, because GH may be produced in extra-pituitary tissues, it is possible that early embryonic/foetal growth and development may be regulated by extra-pituitary GH, in autocrine/paracrine or even endocrine modes of action.

2. Somatotroph Ontogeny

Most immunohistochemical studies indicate that pituitary somatotrophs in the chicken pituitary are not detectable until around mid embryogenesis (ED 10/12) when they may account for as little as 0.2% of all parenchymal cells. During ED 12 -20 of incubation, somatotrophs account for <3.6% of the anterior pituitary cell population; at hatch they account for >20% of the anterior pituitary cell population. Secretory granules are small and under-developed at this point and mature granules are not detectable until ED 15 (Jozsa *et al.* 1979; Malamed *et al.* 1993). It is therefore unlikely that embryonic growth and development are dependent upon pituitary GH.

There is, however, some controversy regarding the initial appearance of GH-immunoreactive somatotrophs in the chick embryo. Some studies have reported the initial appearance of GH-immunoreactive somatotrophs as early as ED 7-8 (Gasc and Sar, 1981; Mikami and Takahashi, 1987). Furthermore, Thommes *et al.* (1987) reportedly found GH-immunoreactivity in 24% of all cells in the Rathke's pouch (the pituitary precursor) on ED 4.5. It is, nevertheless, unlikely that these cells secrete GH until much later in development (Porter *et al.* 1995a). Porter (1997) stated that the demonstration of GH-secreting cells was incredulous, as the Rathke's pouch is little more than a rudiment at this stage. Instead, Porter suggests that functional GH-secreting cells are not present until mid-embryogenesis. Porter *et al.* (1995a; 1995b) detected GH-releasing somatotrophs by reverse hemolytic plaque assay (RHPA), which detects hormone secretion from individual cells in culture. No GH-secreting

cells were detected on ED 10 and on ED 12 only an occasional GH-releasing cell was detectable; by ED 14 the number of GH-releasing cells had increased, but still only account for 2% of all pituitary cells and was still not statistically different from ED 10. The GH-releasing cell population accounts for 6% of pituitary cell population by ED 16, at an abundance statistically significant from ED 10. These results indicate that differentiation of pituitary somatotrophs occurs in large part by ED 16.

The late ontogeny of GH-secreting cells in the chick embryo is also supported by studies on GH synthesis in the embryonic pituitary. The synthesis of GH in the embryo is thought to be minimal and restricted to the latter half of development (Porter, 1997). GH gene transcripts are present in ED 12 embryos, but the abundance of these transcripts, even at ED 18, is <20% of that found in newly hatched birds, and 3% of that found in 4 week old chicks (McCann-Levorse *et al.* 1993). Furthermore, the the appearance of GH in plasma of chick embryos is tightly correlated with somatotroph ontogeny and GH synthesis (Kansaku *et al.* 1994), suggesting a link between somatotroph differentiation, GH synthesis and GH release.

3. Pituitary Somatotroph Differentiation

It is not until ED 16 that a significant number of GH-secreting cells are present in the chick embryo. Peak concentrations of the pituitary-specific transcription factor-1/growth hormone factor-1 (PIT-1/GHF-1) are observed on ED 15, immediately before functional GH secreting cells are present in the pituitary

(Scanes, 1997). It is well known in mammals that PIT-1 is responsible, at least in part, for the modulation of gene expression for several anterior pituitary hormones (Porter, 1997). It seems likely then, that the differentiation of GH-secreting cells in the chick pituitary gland are dependent upon this transcription factor.

Porter and his colleagues (1995a,b) examined the possibility that the ontogenic appearance of somatotrophs was, however, a result of extracellular signals. They determined the ability of unstimulated chick pituitary cells to differentiate into somatotrophs during extended culture. Anterior pituitary cells from ED 10, 12, 14 and 16 embryos were cultured for 2 or 6 days in a defined serum-free medium; no cells were stimulated to differentiate into somatotrophs from cultures derived from ED 10 pituitaries; very few somatotrophs were present in the other cultures derived from ED 12 and 14, even after 6 days of culture. However, a significant proportion of somatotrophs were present in cultures derived from ED 16 pituitaries. These data indicate that anterior pituitary cells will not differentiate autonomously without prior stimulation by an extracellular signal that is only present after ED 14. Therefore, Porter and his colleagues, began to look for an extracellular blood-borne signal which regulated somatotroph differentiation. They collected serum from ED 12 and ED 16 embryos and applied this to ED 10, 12, 14 and 16 anterior pituitary cell cultures. Cells from ED 10 embryonic pituitaries were not responsive to the serum from either ED 12 or ED 16 embryos. Cells from 12, 14 and 16 were responsive to serum from ED 16 embryos and this resulted in an increase in the proportion of GH-secreting

somatotrophs. Results were similar between 2 and 6 days of culture and serum from ED 12 did not significantly increase the proportion of GH-secreting cells in culture.

These results suggest that an inducing factor present in serum of ED 16 embryos is capable of differentiating pituitary cells into functional somatotrophs. This signal is thought to be a glucocorticoid hormone, since corticosterone similarly induced somatotroph differentiation, and its plasma concentration increases between ED 12 and 16 in the chick embryo (Porter *et al.* 1995a; b). It seems unlikely, therefore, that secretion of pituitary GH into systemic circulation occurs before ED 16 of chick embryonic development.

4. Ontogeny of Plasma GH

In the chicken, plasma GH concentrations show a monophasic ontogenic profile, characterised by an increase in late embryonic development and early post hatch growth. Plasma GH is largely undetectable until the end of embryonic development in the chicken. Prior to ED 16, plasma GH concentrations are very low and are probably too low to have physiological effects (Table 1.2). Harvey *et al.* (1979) found that GH was not detectable until ED 17 in serum and serum concentrations of GH increased from ED 17 through to the day of hatch, probably reflecting the parallel increase in the number and pituitary content of somatotrophs (Mikami, 1996). Similar patterns of plasma GH have also been seen during the embryogenesis of turkeys (Proudman and Wentworth, 1980).

5. Ontogeny of Pituitary GH mRNA

Pituitary GH mRNA in chickens is first detected in late embryogenesis (ED 18: McCann-Levorse *et al.* 1993; ED 16: Kansaku *et al.* 1994) and levels rise until early posthatch life, reaching a maximum at 4 weeks (McCann-Levorse *et al.* 1993) and this is consistent with the ontogeny of pituitary GH content and plasma GH in chickens (Scanes, 1987b). This would suggest that the perihatching elevation in plasma GH corresponds to increased GH content and hence, GH available for release.

6. The GH - IGF-1 Axis

The hypothalamo-pituitary GH - IGF-1 growth axis is well defined in birds at varying ontogenic stages and physiological states. However, the axis is not thought to be functional during embryonic growth in the chick embryo (Scanes, 1997).

In the chick embryo, IGF-1 is produced as early gastrulation (Haselbacher *et al.* 1997). IGF-1 receptor genes are expressed within the first day of chick embryonic development (Scavo *et al.* 1991) and plasma IGF-1 levels are detectable on ED 6 (Robcis *et al.* 1991), well before the synthesis and secretion of GH by the pituitary gland. Furthermore, in late embryonic stages, the level of plasma IGF-1 does not correlate with circulating levels of GH (McGuinness and Cogburn, 1990; Kikuchi *et al.* 1991; Burnside and Cogburn, 1992; Tanaka *et al.* 1996; Radecki and Scanes, 1997), indicating the immaturity of the GH-IGF-1 axis in embryos. IGF-1 mRNA is

readily detectable in most embryonic tissues during mid-late embryogenesis, however, it is not detectable in the liver until ED 18 (Serrano *et al.* 1990; Burnside and Cogburn, 1992; Tanaka *et al.* 1996), suggesting that GH is unable to induce the expression of hepatic IGF-I until the end of chick incubation.

7. Hepatic Growth Hormone Receptors

The endocrine actions of pituitary GH on the growth of postnatal vertebrates are mostly mediated by hepatic IGF's, after the binding of GH to hepatic membrane receptors (Scanes, 1987). Hepatic GHRs are, however, not detectable until the third trimester of embryonic development in the chick, and hepatic GHR mRNA, is first detectable only after ED 15 (Burnside and Cogburn, 1992; Tanaka *et al.* 1996). Furthermore, hepatic binding sites are not demonstrable until ED 14 (Vanderpooten *et al.* 1991; Vanderpooten *et al.* 1992) and it is therefore unlikely that pituitary GH has any roles in the growth of the early-mid stage embryo.

8. Experiments with Hypophysectomized Embryos

The possibility that embryonic/foetal growth and development may be independent of pituitary GH is further supported by studies on hypophysectomised/decapitated embryos. Chick embryos decapitated at 33-38h grow normally (as measured by dry body weight) until ED 11.5 (Thommes and Woods, 1993). It is therefore axiomatic that early embryonic growth is pituitary GH-

independent. Furthermore, the chronic treatment of pituitary-deficient chick embryos with chicken GH, in the latter half of the 21 day incubation period, does not result in any increased growth (Thommes *et al.* 1992). Likewise, exogenous GH has been found to have no effect on the body weight of normal chick embryos injected between ED 6-14 (Murphy *et al.* 1986), on ED 11 (Hargis *et al.* 1989), or ED 13 (Darras *et al.* 1992). Similar results have also been reported for turkey embryos (Maruyama *et al.* 1996).

9. Mammalian Foetal Growth

Mammalian foetal growth is similarly independent of pituitary GH. Indeed, Jost and coworkers intensely studied the hormonal regulation of foetal growth in rats and rabbits and concluded that the removal of pituitary GH by decapitation has little effect on total body growth (see Thommes and Woods, 1992 for review). The foetal decapitation of pigs has also shown that increases in foetal body weight are independent of pituitary regulation (Fentener van Vlissingen *et al.* 1983; McCusker and Campion, 1990).

D. GH Dependent Action in the Embryo

1. Introduction

It has been generally thought that pituitary GH has no growth-promoting effects in the vertebrate embryo (Scanes, 1987b), although data from the 1950s

showed that crude GH preparations could increase the body weight of chicken embryos (Blumenthal *et al.* 1954). This observation was confirmed in more recent studies with purer GH preparations, which also increased liver weight and induced skeletal growth (Thommes *et al.* 1992). Furthermore, the presence of GHRs in early embryonic tissues (Table 1.3) suggests GH has actions during chick embryogenesis. Indeed, it is now clear that there is a plethora of GH dependent actions in embryonic chickens (Table 1.4).

2. Growth and Differentiation

There are several lines of evidence which suggest that GH may possess some growth-promoting activity in chick embryos. Thommes *et al.* (1992) demonstrated that purified cGH was able to partially restore tibial length in decapitated embryos. In this study, surgical decapitation of the embryo at ED 1.5 resulted in a decrease in body and liver weights and in skeletal growth as indicated by tibial length. A pituitary gland transplanted onto the chorioallantoic membrane partially restored torso growth. However, this impeded growth probably reflects the reduced ability of the embryo to ingest albumen which is markedly affected by decapitation (Betz, 1968).

There is evidence from the 1950s that GH stimulates skeletal growth and increases hatch weights of chicks administered mammalian GH during embryonic development (Blumenthal *et al.* 1954; Hsieh *et al.* 1952). Furthermore, *in ovo* administration of ovine (o) GH was shown to increase body weight and skeletal

growth of male broiler chickens at 7 weeks of age (Hargis *et al.* 1989). This contrasts with previous studies suggesting that female broilers are more sensitive to GH administration (Hsieh *et al.* 1952) than their male counterparts.

Administration of oGH to turkey embryos on ED 14 resulted in increased shank length and increased weights of gastrocnemius and the sartorius muscles (Maruyama *et al.* 1996) in 4 week old birds. Studies of this nature raise the possibility that embryonic GH could influence physiology later on in development. Hormonal imprinting is a delayed physiological response to an episode of hormonal excess in embryos (Csaba, 1986).

3. Other effects

GH increases plasma concentrations of T_3 and decreases T_4 in chick embryos by influencing the activities of the enzymes responsible for hepatic monodeiodinasation and hepatic type II deiodinase activity (Kuhn *et al.* 1988; Darras *et al.* 1993).

In decapitated embryos, GH increases the content and concentration of DNA in the liver (Thommes *et al.* 1992) and tissue levels of RNA (Wang *et al.* 1953). GH has also been shown to stimulate thymidine uptake and DNA synthesis in chicken foetal osteoblasts in culture (Meier and Solursh, 1972).

Administration of cGH increases blood sugar levels in chick embyros (Hsieh *et al.* 1952), suggesting GH is able to influence the regulation of blood glucose. GH

also reduces allantoic concentrations of sodium [Na^{2+}] at ED 10 and ED 14 in chick embryos (Murphy *et al.* 1986).

In a recent study, Gould *et al.* (1995) showed that anterior pituitary gland grafts onto the chorioallantoic membrane (CAM) of chick embryos evoked an angiogenic response. They then studied the effect of GH and it was shown to increase the number of blood vessels on the CAM treated with cGH or bovine (b) GH. It is possible that GH may be acting via IGF-I as it is capable of acting as an angiogenic agent in mammals (Grant *et al.* 1993).

8. GH effects in mammalian development

As in birds, abundant evidence exists that GH possesses biological activity in the mammalian foetus. It has been postulated to have effects in sexual differentiation, growth and cellular differentiation, and cellular metabolism. Furthermore, the recent demonstration of GH and functional GHR expression in preimplantation murine embryos (Panteleon *et al.* 1997), suggests that GH possesses metabolic roles in the earliest stages of embryogenesis.

It was shown recently that immunoreactive GH and GHR were expressed in developing rat tooth germs, well before the pituitary gland became competent (Zhang *et al.* 1997). GH-immunoreactivity was observed in ontogenic cells undergoing differentiation in a cell-type and developmental stage-specific pattern, and these

results support earlier studies implicating GH in rat (Zhang *et al.* 1992a; Zhang *et al.* 1992b; Young, 1995) and mouse foetal dentogenesis (Young, 1995).

GH-immunoreactivity was detected in the foetal mouse reproductive tract and this material increased in concentration in response to progression of sexual differentiation (Nguyen *et al.* 1996). Furthermore, it was noted that GH influenced the androgen-binding activity of the reproductive tract. Anti-GH serum prevented the development of the Wolffian duct and this was reversible with exogenous GH administration. IGF-I was also capable of reversing this affect in culture experiments and therefore it is possible that GH is acting via local IGF-I.

Recent work has shown the expression of GH and GHR in the preimplantation murine embryo (Panteleon *et al.* 1997). Furthermore it was shown that these receptors were functional, as exogenous GH influenced glucose uptake and protein synthesis. Clearly, GH is expressed in embryos before the development of the pituitary gland, and is likely to have roles in embryonic/foetal growth and development.

VI. Summary

The growth and development of vertebrate embryos is a very complex process involving many different processes and growth factors and the role of GH in these phenomena is unclear. Furthermore, there are significant differences between these processes in mammalian foetuses and avian embryos, which have been primarily used as experimental models. For instance, IGF-II is a major component of the mammalian foetal "growth" system but it appears to have no role in avian embryogenesis (Thommes and Woods, 1993).

Embryonic or foetal growth has long been considered to be independent of the actions of pituitary GH. A possible reason for this is that GH is only one factor of many which are contributing to the regulation of embryonic/foetal growth. This may explain why GH does not consistently increase total body growth, but does possess biological activity within the embryo/foetus. Some of these effects are concerned with the growth of organs, of cellular differentiation or proliferation or in metabolic activities. GH is produced by numerous tissues in postnatal vertebrates and it is likely that the situation is similar in embryonic tissues, especially as GH is expressed in preimplantation murine embryos. Therefore, it is likely that GH is acting as a paracrine or autocrine to regulate certain aspects of development and this hypothesis is supported by the presence of GHR's in prenatal tissues.

VII. Hypothesis and Specific Aims

This thesis is based upon the abundant literature which provides evidence that GH is produced in many extra-pituitary sites in postnatal animals. Additionally, GH has many effects with avian embryos and we hypothesised that GH may be produced in extra-pituitary tissues of the chick embryo. The specific amis were:

1). To establish the distribution of extra-pituitary GH expression during early chick embryogenesis

2). To establish whether the GHR is also expressed in these tissues

The work of this thesis has relevance to the growing body of data which suggests that hormones are not only produced within discrete endocrine glands but are produced in a multitude of tissues, where they may possess roles not directly related to their "major" physiological function. The results of this thesis also challenge the dogma that GH is not involved in embryonic or foetal growth and development

Table 1.1. Sites of Extrapituitary Growth Hormone (GH)

Species	Tissue	GH	GHmRNA
FISH	neurohypophysis	Hansen and Hansen, 1982	
	brain	"	
FROG	brain	Yon *et al.* 1991	
CHICKEN	brain	Render *et al.* 1995a	Render *et al.* 1995a
"	spleen	Render *et al.* 1995b	Render *et al.* 1995b
"	Mullerian duct	Wang, 1989	
RAT	neurohypophysis	Lechan *et al.* 1983	
"	brain	Hojvat *et al.* 1982	Martinoli *et al.* 1991
"	spinal cord	Lechan *et al.* 1981	
"	kidney	Kyle *et al.* 1981	
"	lung	"	
"	GIT	"	
"	placenta	Ogilvie *et al.* 1990	
"	Wolffian duct	Nguyen *et al.* 1996	
DOG	mammary gland	Mol *et al.* 1995	Mol *et al.* 1995
CAT	"	"	"
SHEEP	pineal gland	Noteborn *et al.* 1993	
	placenta	Scippo *et al.* 1992	Scippo *et al.* 1992
MONKEY	placenta		Golos *et al.* 1993
HUMAN	testis		Untergasser *et al.* 1996
"	mammary gland	Mol *et al.* 1996	Mol *et al.* 1996
"	placenta	Cooke and Liebhaber, 1997	Cooke and Liebhaber, 1997
"	muscle	Kyle *et al.* 1981	Wu *et al.* 1996
"	skin		Palmetshofer *et al.* 1995
"	thymus	Sabarwal and Varma, 1996	Wu *et al.* 1996
"	tonsils		"
"	lymph node		"
"	spleen		"
"	lymphocytes	Varma *et al.* 1993	"

GIT, gastrointestinal tract

Table 1.2. Ontogeny of Plasma Growth Hormone (GH) in the Chick Embryo

ED[1]	Plasma GH (μg/L)	Reference
9-11	<1	Kikuchi *et al.* 1991
12-15	2.3	"
16	5.0	"
17	9.0	"
18	7.0	"
19	7.5	"
20	6.0	"
21	40.0	"
9-15	<5	Harvey *et al.* 1979
17	7.5	"
19	10.0	"
18	<10	Berghman *et al.* 1989
15-19	<5	McGuinness and Cogburn, 1990
21	8.4	"
14-20	<5	Darras *et al.* 1994
18	4-5	"
20	6-8	"
21	35.7	"
17-21	25-30	Decuypere and Scanes, 1983
19	<10	Hoshino *et al.* 1990
16	<6	Huybrechts *et al.* 1990
18	<10	Huybrechts *et al.* 1985
20	<10	"
18	<10	Kuhn *et al.* 1988
16-20	<15	Vanderpooten *et al.* 1992
18	<10	Geris *et al.* 1996

[1] **ED, embryonic day in the 21 day incubation period**

Table 1.3. Growth Hormone (GH) and Growth Hormone Receptor (GHR) in embryonic/foetal tissues

Species	Tissue	GH	GH mRNA	GHR	GHR mRNA
CHICKEN	endoderm	Wang, 1989			
"	ectoderm	"			
"	reproductive tract	"			
MOUSE	pre-implantation embryo (blastula)	Panteleon *et al.* 1996	Panteleon *et al.* 1996	Panteleon *et al.* 1996	Panteleon *et al.* 1996
"	embryonic stem cells				Ohlsson *et al.* 1993
	reproductive tract	Nguyen *et al.* 1996		Ohlsson *et al.* 1993	"
RAT	brain			Garcia-Aragon *et al.* 1992	
"	hypothalamus			Hasegawa *et al.* 1993	
"	muscle			"	
"	chondrocytes			"	
"	GIT			"	
"	lung			"	
"	kidney			"	Chin *et al.* 1992
"	ovary			"	
"	reproductive tract			"	
"	dermal fibroblasts			"	
"	lens			"	
"	dental papilla	Zhang *et al.* 1997		Zhang *et al.* 1997	
				Joseph *et al.* 1994	
"	enamel organ			"	
"	odontoblasts			"	
"	ameloblasts			"	
"	dental mesenchyme			"	
RABBIT	chondrocytes				Barnard *et al.* 1988
"	muscle				Ymer and Herington. 1992
"	heart				"
"	kidney				"
COW	brain				Scott *et al.* 1992
"	spleen				"

Table 1.3. (cont)

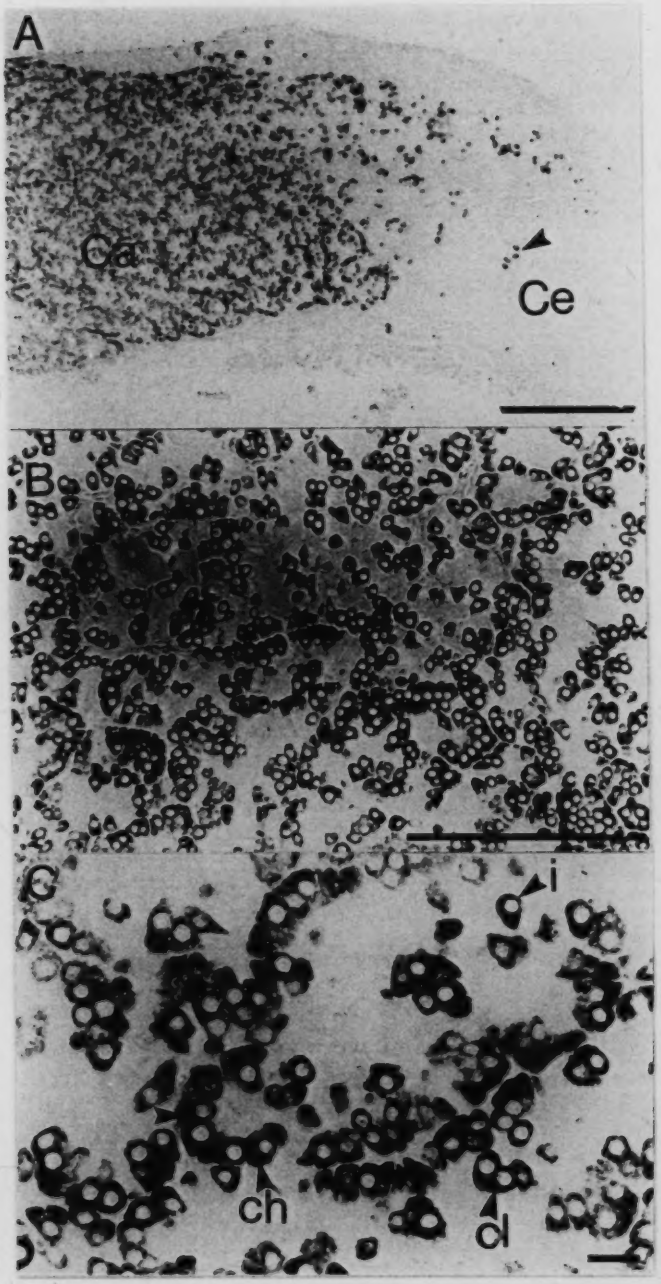
Species	Tissue	GH	GH mRNA	GHR	GHR mRNA
"	thymus				"
"	kidney				"
SHEEP	brain				
"	muscle				Klempt *et al.* 1993
					Klempt *et al.* 1993
PIG	muscle				
"					Duchamp *et al.* 1996
"	muscle				
					Bingham *et al.* 1994
HUMAN	brain			Hill *et al.* 1992	
"	muscle				
					Wu *et al.* 1996
"	chondrocytes				Simard *et al.* 1996
"	spleen				Werther *et al.* 1993
"	thymus				Wu *et al.* 1996
"	lung			Hill *et al.* 1992	
				"	
"	kidney			Wu *et al.* 1996	
				Hill *et al.* 1992	
"	adrenal glands			Simard *et al.* 1996	
"	GIT*			"	
"	vascular endothelial cells			"	
"	skin			"	
					Werther *et al.* 1993

*GIT, gastrointestinal tract

Table 1.4. Growth Hormone (GH) Actions in the Chick Embryo

Species	Action	Reference
ED 10 chick	Thymidine incorporation in to cartilage	Jennings *et al.* 1980
ED 10 chick	Sulphate incorporation into cartilage	"
ED 11 chick	Reduction of allantoic [Na^+]	Murphy *et al.* 1986
ED 16.5 chick	Increased liver DNA content + concentration	Thommes *et al.* 1992
ED 18 chick	Increased plasma T_3; decreased plasma T_4	Darras *et al.* 1992; 1993
ED 18 chick	Increased conversion of T_4 into T_3	Berghman *et al.* 1989

Fig 1.1. Growth hormone immunoreactivity in pituitary somatotrophs. GH is produced by somatotrophs in the adult pituitary gland. A: Somatotrophs are largely confined to the caudal lobe (Ca) of the anterior pituitary although some isolated cells and small groups are located in the cephalic lobe (Ce). Bar = 500μm. B: Higher power of A showing GH-immunoreactive somatototrophs. Bar = 200μm. C: Higher power of B showing that somatotrophs exist as either isolated cells (I), clumps (cl) of around 3-5 cells or as chords (ch) of 5 or more cells. Bar = 10μm. This figure is the author's work.


A
Ca
Ce
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C
i
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Chapter 2

Extra-pituitary Growth Hormone in the Early Chick Embryo

Some of the results in this chapter were presented at the 6th International Symposium on Avian Endocrinology (1996), Lake Louise, Canada (Abstract 3.12) and the 5th Western Perinatal Research Meeting (1997), Banff, Canada (Abstract 8.12). Data from this chapter were also published in C.D.M.Johnson, M.A. Wride, K.L. Hull, and S. Harvey (1996). Immunohistochemical Localization of Growth Hormone and Growth Hormone Receptor in the Early Chick Embryo. *Poultry and Avian Biology Reviews*, Vol 6, 4: 259, and S. Harvey, C.D.M. Johnson, P. Sharma, E.J. Sanders and K.L. Hull (1998). Growth Hormone: An Embryonic Paracrine? *Comparative Biochemistry and Physiology,* (in press).

I. INTRODUCTION

The growth and development of the embryo or foetus is generally considered to be independent of pituitary growth hormone (GH) and the embryonic or foetal period is viewed as a period of GH deficiency (Geffner, 1996). Indeed, early development occurs prior to the differentiation of pituitary somatotrophs and the ontogenic appearance of GH in peripheral plasma, since pituitary GH is not detectable until mid-late embryogenesis or gestation in all vertebrate groups (Thommes and Woods, 1993). In the chicken, for instance, the pituitary gland does not differentiate until day 7 of the 21 day incubation period and GH-secreting cells are not present until day 16 (Porter, 1997). Furthermore, while circulating GH is detectable on day 17 (Harvey *et al.* 1979), pituitary GH mRNA is not measurable until day 18 (McCann-Levorse *et al.* 1993). Approximately 80% of chick embryonic development, therefore, occurs in a period of pituitary GH deficiency. Early embryos may, moreover, be resistant to the growth-promoting actions of pituitary GH during this period.

It is well established that postnatal growth and development are critically dependent upon pituitary GH but early embryogenesis is likely to be pituitary GH independent. Indeed, the removal of the pituitary gland by decapitation, at 33-38 hours of incubation, does not impair the ability of chick embryos to grow normally (as measured by dry weight) until mid-incubation (Thommes *et al.* 1992). Furthermore, the treatment of headless (Tixier-Vidal, 1954) and intact (Hargis *et al.*

1989; McCann-Levorse *et al.* 1993) embryos with GH does not affect total body weight. Exogenous GH similarly fails to induce chondrocyte proliferation in chick embryos (Halevy *et al.* 1996; Monsonego *et al.* 1995). This may reflect the inability of pituitary GH to induce the hepatic production of insulin-like growth factors (IGFs) during early embryonic development. These growth factors are inducible during postnatal development and are thought to mediate endocrine actions of pituitary GH on the musculo-skeletal system (Scanes, 1997). The liver is the principle site of IGF production postnatally but it lacks GH receptors until day 15 of chick embryogenesis (Burnside and Cogburn, 1992; Vanderpooten *et al.* 1992). The late ontogeny of hepatic GHR is similarly thought to account for the inability of pituitary GH to induce early foetal growth in mammals (Gluckman *et al.* 1983; Breier *et al.* 1989).

The pituitary is the primary site of postnatal GH expression, but it also occurs in neural (Harvey *et al.* 1993), immune (Render *et al.* 1995b) and reproductive tissues (Garcia-Aragon *et al.* 1992; Nguyen *et al.* 1996; Untergasser *et al.* 1996) and in skin (Palmetshofer *et al.* 1995) and blood endothelial (Brigham *et al.* 1993) cells. Extra-pituitary GH is, however, unlikely to be released into systemic circulation, since plasma GH concentrations are undetectable following hypophysectomy (Meier and Solursh, 1972; Harvey and Scanes, 1977; Leung *et al.* 1986). Extra-pituitary GH may, however, act as a paracrine or autocrine growth factor (Harvey and Hull, 1997), especially since GH receptors are present in many tissues during foetal development before the ontogeny of hepatic GHRs (Burnside and Cogburn, 1992). It is therefore

possible that extra-pituitary GH may act as a local growth factor during embryonic development. This possibility is supported by the recent demonstration of GH and GHR gene expression in preimplantation mouse embryos, in which exogenous GH stimulated glucose transport and protein synthesis (Panteleon *et al.* 1997). Extra-pituitary GH production during organogenesis has, however, yet to be determined in any species. The possibility that GH may be produced by extra-pituitary tissues during early embryonic development was therefore assessed in chick embryos, in which the ontogeny of organogenesis is well described (Lillie, 1952; Romanoff, 1960; Patten, 1971).

II. MATERIAL AND METHODS

Tissues

Fertile White Leghorn eggs (University of Alberta farm) were incubated at 37.5°C in humidified air (Hamburger and Hamilton, 1951). The eggs were turned one quarter of a revolution each day during incubation. Whole chick embryos at embryonic day (ED) 3, 4, 6, 7, and 8 were dissected in phosphate buffered saline (PBS; pH 7.4). Older embryos which were too large for fixation and sectioning were cut into head, torso and limbs. Chick embryos were sacrificed according to the guidelines outlined by the Canadian Council of Animal Care (1993).

Immunocytochemistry

Tissues were fixed in paraformaldehyde (4% w/v) (Sigma, Ontario, Canada) or Bouin's fixative overnight at 4°C. Tissues were then dehydrated in a graded series of alcohol (50%, 15-30 mins; 70%, 30-60 mins; 95%, 30-60 mins; 100%, 60-120 mins) (dehydration times were lengthened relative to tissue size) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 30 mins. Tissues were then infiltrated with paraffin wax for 24-48 hours at 60°C, under normal atmospheric pressure.

Serial transverse (4-8μm) sections were cut through the whole embryo or embryonic tissue using a microtome and mounted onto charged slides (Fisher Scientific, Canada). Immunocytochemical staining was performed with commercial

reagents (Vector Laboratories, CA, USA; Sigma, Canada) using the avidin-biotin-peroxidase (ABC) (Hsu *et al.* 1981) or the alkaline phosphatase, anti-alkaline phosphatase (APAAP) (Sternberger, 1979) method. The ABC technique was, however, mostly used because of its greater sensitivity and simplicitly of use. Sections were incubated with specific polyclonal antisera raised in rabbits against chicken (c) GH (αcGH-1: Harvey and Scanes, 1977; αcGH-2: Porter *et al.* 1995a). Both of these antibodies were diluted 1:4000 in PBS or 1-5% Normal Goat Serum (NGS) overnight at room temperature (RT). A mouse monoclonal antiserum raised against glycosylated chicken growth hormone (IH7: Berghman *et al.* 1987) was also used at 1:1000 diluted in NGS or PBS. After incubation the slides were washed 3 times for 15 mins in PBS. Sections were then incubated in biotinylated goat anti-rabbit IgG (Sigma) (1:500) for 1 hour at RT, for the polyclonal antibodies, or in a biotinylated anti-mouse IgG (Sigma) for the monoclonal antibody. Slides were then washed as before in PBS. Finally, sections were incubated in ABC reagent for 1 hour at RT and washed as before. Staining was visualized using the chromogenic substrate diaminobenzidine tetrahydrochloride (DAB) (Sigma), which resulted in a brown colouration. In some instances nickel chloride (Sigma) was added to the DAB, resulting in a black precipitate. Specificity of staining was determined by preabsorbing the GH antisera with recombinant cGH (Amgen, Thousand Oaks, CA, USA; 1mg/ml) for 1 hour prior to section incubation. Antibody specificity was also shown by cross-reactivity with chicken somatotrophs (Fig 1.1). Non-specific staining

was determined by replacing GH antisera with pre-immune rabbit serum. Other controls included the omission of the secondary antibody and replacing the primary antibody with PBS.

The APAAP technique involved the use of a secondary antibody conjugated with alkaline-phosphatase (Sigma, Canada). After incubation with the secondary antibody, the sections were washed, as outlined above, and incubated with APAAP diluted 1:10 for 1 hour at RT. The sections were then washed and Fast Red TR/Napthol As-MX (Sigma, Canada) was applied to tissues sections until optimum colour development.

GH receptor (GHR) immunoreactivity was detected using a polyclonal antibody raised against cGHR (Huang *et al.* 1993), using the ABC technique, as above.

Rationale for tissue choice

This is a preliminary study and a range of embryonic ages were chosen to be studied. When promising results were obtained from a particular tissue, then study was focused upon this tissue. Any following study should attempt to provide a more complete ontogenic map of GH and GHR distribtion.

Western analyses

Pooled tissues or whole embryos (from approximately 5-10 embryos) were rapidly dissected from eggs in PBS. They were then immediately homogenised (1g/10 ml) in a solution containing 1% (w/v) SDS, 1mmol PMSF/l and 10 ug aprotinin/l, using a Polytron homogenizer (Brinkman Instruments, Westbury, NY, USA). Homogenates were centrifuged at 200 g for 5 min at 4°C and protein was diluted 1:1 with loading buffer (0.06M Tris.HCl pH 6.8; 10% glycerol; 2% SDS; 5% 2-β-mercaptoethanol; 0.001% bromophenol blue) and heated to 55°C for 15 min prior to loading. Proteins were then separated by electrophoresis in 15% SDS-polyacrylamide gels under reducing conditions. Gels were then equilibrated in transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) and transferred electrophoretically (30V, 4 hours at 4°C) to Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Non-specific binding sites were blocked by incubating in 5% skimmed milk, dissolved in Tris buffered saline (25nM Tris.HCl; ph 7.5; 0.5M NaCl) at RT for 1 hour. GH immunoreactivity was detected using a rabbit polyclonal antibody (αGH1: Harvey *et al.* 1979), diluted 1:1000 in TBS/5% skimmed milk and incubated with the membrane overnight at room temperature. Membranes were incubated with a horseradish peroxidase conjugated anti-rabbit IgG (Amersham or Sigma) diluted 1:2000 in TBS/5% skimmed milk. After washing, blots were developed with an enhanced chemiluminescence detection system (ECL kit, Amersham) for 1 min.

Membranes were exposed to Kodak X-AR film (Kodak, Rochester, USA) for 30 seconds to 1 hour.

Polymerase chain reaction *(carried out by P. Sharma)*

The presence of GH mRNA was assessed using the reverse transcribed polymerase chain reaction (RT-PCR). Pooled total tissue RNA (from 5-10 embryos), from the bodies (without the heads) of ED 7 embyros, was reversed transcribed in the presence of 10 pmol of the oligo-deoxythymine GH primer, khu 14, 5'-GACTCGAGTCGACAT CGTTTTTTTTTTTTTTTT-3', (Nucleotide Synthesis Laboratory, University of Alberta), 5X RT buffer (Promega), excess deoxynucleotides (10nM each of deoxy -ATP, dCTP, dGTP and dTTP, (Boehringer Mannheim, Quebec, Canada), the reverse transcription enzyme Superscript (100 U, BRL, Burlington, Canada) and 0.1 m dithiothreitol (DTT). This mixture was incubated at 42°C (1 hour) and the generated cDNA was diluted in 480µl double-distilled water. For comparative purposes, reverse transcribed RNA from adult chicken pituitary glands was used as a positive control. Tissue RNA not reverse transcribed (mixture same as above but lacking Superscript) served as a negative control for the experiment. The cDNA was amplified in the presence of oligonucleotide primers GH 3' (antisense) and GH 5' (sense) (GH 3', 5'-GCCTCAGATGGTGCAGTTGCTCTCTCCGAA- 3'; GH 5', 5'-CGTTCAAGCAAC-ACCTGAGCAACTCTCCCG-3', Nucleotide Synthesis

Laboratory, University of Alberta), excess deoxynucleotides (1.25 mM of each), 10 X PCR buffer, 25 mM MgCl and Thermus aquaticus (TAQ) DNA polymerase (5 U, Promega). The primers were designed to generate a 689 base pair (bp) fragment spanning the entire coding region of the cGH gene (see Hull *et al.* 1996 for details). The reaction mixture was then overlayed with 2 drops of mineral oil to prevent evaporation. The samples were denatured at 92°C (3 minutes) after which they were exposed to 30 cycles of denaturing (92°C for 1 minute), annealing (60°C for 1 minute), extension (72°C for 2 minutes) and then a final extension (72°C for 10 minutes) in thermal cycler (MJ Research, Watertown, MA, Canada). The amplified cDNA (15µl) was then electrophoresed in an ethidium bromide-stained agarose gel (1.2% wt/vol) and viewed under ultra-violet light (UV).

The presence of GHR mRNA was also assessed by RT-PCR. Total cellular RNA from whole embryos (ED 1, 3, 4, 5) was pooled from at least 10 embryos. Total cellular RNA from headless bodies of ED 7 and 9 embryos was pooled from 4-7 embros. Total cellular RNA (pooled from 5 embryos) was also obtained from ED 18 livers. All RNAs were reverse transcribed and amplified by 30 cycles of PCR in the presence of oligonucleotide primers GHR_3 (TAAACTTGTAAGAACCACGG) and K12 (details in Hull *et al.* 1996), which amplify a 1100 bp fragment encoding the intracellular domain of the GHR (Burnside *et al.* 1991). The methods and chemicals used were identical to those above. Amplified cDNA moieties were visualised by electrophoresis in 1.5% ethidium-bromide stained gels. For comparison, adult liver

that had been reverse transcribed in the absence and presence of reverse transcriptase acted as negative and positive controls respectively.

III. RESULTS

Immunocytochemistry

The distribution of GH-immunoreactive cells in the tissues of embryonic chicks between days 3 and 8 of incubation is summarised in Table 2.1.

At ED 3 GH-immunoreactivity was ubiquitous in transverse tissues sections taken through the mesonephros (Fig 2.2 A and C) or through the developing heart (Fig 2.2 E) of the embryo. These sections are representative of staining seen in all levels of the ED 3 embryo. Control sections incubated with NGS or PBS were unstained (data not shown). Incubation with αGH1 (Fig 2.2 A), αGH1 (Fig 2.2 C) or EH7 (Fig 2.2 E) intensely labeled all tissues. The specificity of this staining was demonstrated by its complete abolition following the preabsorption of the primary antisera with recombinant cGH (Fig 2.2 B, D and F).

At ED 5 GH immunoreactivity was observed in the developing kidney and reproductive system (Fig 2.3). The Wolffian duct was more intensely stained than adjacent tissues (kidney tubules and body mesoderm) (Fig 2.3 C and D), and a single layer of densely stained cells is seen in the Wolffian duct (Fig 2.3 D). GH immunoreactivity was not, however, present in all of these cells. Preabsorption of

αGH1 with recombinant cGH completely abolished this staining (Fig 2.3 B). Similar results were also observed with αGH2 (data not shown).

Cells immunoreactive with αGH1 were widespread in the 6 day embryo (Fig 2.4 A) with the exception of the notochord. Strong staining was present in the neural tube, myotome, limb bud mesencyme, amnion, developing kidney, and in mesoderm throughout the body. Preabsorption of the αIH7 with recombinant cGH did not result in staining (Fig 2.4 B). The staining was not in every cell, as indicated by the immunoreactivity of embryonic mesoderm (Fig 2.4 C). Although these irregular shaped cells appear to be morphologically homogeneous, some cells showed lighter staining or totally lacked staining (arrows).

The ED 6 amnion or extra-embryonic membrane showed an interesting pattern of GH-immunoreactivity. The ectodermal layers of the membrane were strongly immunoreactive (Fig 2.5 A). This was due to non-specific staining since the staining was not in the outer edges of the tissues and because preabsorption of the primary antisera with recombinant cGH greatly reduced staining (Fig 2.5 B). The mesodermal layer of the amnion at ED 6 is beginning to differentiate from a syncitium into muscle tissue. Although the individual cells are not visible at this stage the nuclei of these differentiating cells were very strongly immunoreactive (Fig 2.5 C). The cytoplasm, however, was not immunoreactive. Preincubation of primary antisera with recombinant cGH reduced staining (Fig 2.5 D).

At ED 7 staining was seen in the amnion (Fig 2.6 A), myotome (Fig 2.6 B), endothelial lining of blood vessels and circulating blood cells. (Fig 2.6 C). Rathkes Pouch (data not shown) and diencephalon. It is clear while some blood cells were GH-immunoreactive, others were not (Fig 2.6 C). This suggests that the staining of blood cells is specific. Although scattered mesodermal cells were still immunoreactive, the mesoderm was not as densely stained as in younger stages (Fig 2.6 B) and therefore, the immunoreactivity of the myotome is distinct. The epidermis was also strongly stained for GH using αGH1. Preincubation of αGH1 with recombinant cGH completely abolished immunoreactivity. Fig 2.6 D is shown as a representation of the preabsorption control and is a similar section to Fig 2.6 C.

The ED 8 limb bud was GH-immunoreactive (Fig 2.7). The wing buds were sectioned and stained with αGH1. Immunoreactivity was seen in the cartilage cells of the zone of hypertrophy (Fig 2.7 A). Some cells showed strong cytoplasmic staining or nuclear staining and some cells did not appear to have any immunoreactivity (Fig 2.7 A). GH-immunoreactivity was similarly observed in most cells from the zone of flattened cells undergoing mesenchymal condensation (Fig 2.7 B). Most cells had either cytoplasmic or nuclear staining, although some had both nuclear and cytoplasmic staining, whereas others were totally devoid of GH immunoreactivity. Preincubation of the αGH1 with recombinant cGH abolished staining in cartilage cells (Fig 2.7 D is a similar section to 2.7 A).

Growth hormone receptor-immunoreactivity was seen in a similar distribution to GH-immunoreactivity in the ED 3 embryo (Fig 2.8). Staining was not observed when the antibody was replaced by NRS or PBS. GHR-immunoreactivity was demonstrated in the cartilage cells from the hypertrophic zone in the limb bud from the ED 8 embryo (Fig 2.7 C). GHR-immunoreactivity appeared to be predominantly localised to the nuclei of these cells, although some cytoplasmic staining was observable.

Western analysis

GH-like proteins of approximately 22kDa and 44kDa detected using αGH1 were present in the body of the ED 7 embryo (Fig 2.9). These GH-like proteins correspond to GH moieties in the chicken pituitary gland (Lane 1, Fig 2.9).

Polymerase Chain Reaction

In the presence of oligonucleotide primers for pituitary cGH cDNA (GH_3 and GH_5), a moiety of approximately 689 bp was amplified from the head and body of ED 7 and ED 9 embryos (Fig 2.10). Adult pituitary RNA that had been reverse transcribed in the presence or absence of reverse transcriptase acted as positive and negative controls, respectively.

In the presence of oligonucleotide primers for the intracellular domain of the GHR (GHR_3 and K12), a moiety of approximately 1100bp was amplified from the

whole bodies of ED 1, 3, 4, 5, 7 and 9 embryos (Fig 2.11). Adult liver RNA that had been reverse transcribed in the presence or absence of reverse transcriptase acted as positive and negative controls, respectively.

IV. DISCUSSION

These results clearly demonstrate a widespread distribution of GH-like proteins, similar in molecular size to pituitary GH, in extra-pituitary tissues of early chick embryos. Since pituitary somatotrophs do not secrete GH until ED 16 (Porter *et al.* 1997) and as GH is not detectable in plasma until ED 17 (Harvey *et al.* 1979), this GH immunoreactivity in embryonic tissues cannot result from the sequestration of pituitary GH from peripheral plasma. These GH-like proteins are thus likely to reflect an almost ubiquitous expression of the GH gene in early embryos, as indicated by RT-PCR of extra-pituitary mRNA.

The presence of GH-like proteins in extra-pituitary sites is now well established (Harvey and Hull, 1997) but this is the first study to identify early embryonic tissues that have GH immunoreactivity. Panteleon *et al.* (1997), however, demonstrated that non-maternal GH mRNA was present in preimplantation mouse embryos prior to the formation of the morula. These authors did not determine if GH gene expression persisted during foetal development and thus did not determine the tissue distribution of GH during ontogeny. The present results indicate that all tissues initially express the GH gene, although this capacity appears to be lost in some tissues

(eg. the notochord and mesoderm) by mid-embryogenesis, suggesting an ontogenic extinction of GH gene expression.

The extra-pituitary expression of the GH gene during embryogenesis suggests roles for GH in early embryonic growth or differentiation, especially as the GHR gene also appears to be ubiquitously expressed at this time. It is therefore possible that GH acts as a local growth factor. Indeed, local actions of GH are similarly thought to be responsible for the growth and differentiation of immune tissues and haematopoietic cells (Auernhammer and Strasburger, 1995), and for the growth and differentiation of mammary and placental tissues (Weigent *et al.* 1991; Strowitzki *et al.* 1991), pituitary cells (Gardner *et al.* 1990), fibroblasts (Hauner, 1992) and adipocytes (Catalioto *et al.* 1992). The recent demonstration of GH and GHR immunoreactivity in the teeth of perinatal rats is similarly thought to reflect local actions of GH in chondrocyte development (Zhang *et al.* 1997). This possibility is supported by the demonstration that GH immunoneutralisation blocks Wolffian duct differentiation during foetal rat development, by a mechanism overcome by exogenous GH (Nguyen *et al.* 1996). These putative actions of GH during embryonic development could be direct or mediated by other embryonic growth factors, particularly insulin-like growth factors (IGFs) (De Pablo *et al.* 1990). Although pituitary and plasma GH are not correlated with hepatic IGF-I production during chick embryogenesis (Radecki and Scanes, 1997), GH may induce local IGF production in extra-hepatic sites. The local actions of GH within the mammalian

immune system are known to be at least partly mediated through IGF and IGF-I receptors (see Hull and Harvey, 1997 for review).

The detection of GHR immunoreactivity in early chick embryos was a novel discovery, since hepatic GHR mRNA (Burnside and Cogburn, 1992; Tanaka *et al.* 1996) and radioligand binding sites (Vanderpooten *et al.* 1991; Vanderpooten *et al.* 1992) were not thought to be present until late embryogenesis. This may, however, reflect methodological differences in sensitivity. It may also reflect differences in the ontogeny of GHR expression, since hepatic GHRs appear later during foetal development (Burnside and Cogburn, 1992) than the detection of GHR-immunoreactivity in extra-hepatic tissues in this study. The presence of GHR immunoreactivity in early chick embryos does, however, suggest that the embryo is not GH-resistant. This possibility is also supported by the ability of exogenous GH to induce DNA, RNA and protein synthesis in early chick embryos (Sobel *et al.* 1958; Thommes *et al.* 1992) and to increase blood sugar levels (Hseih *et al.* 1952), to promote angiogenesis (Gould *et al.* 1995), to reduce allantoic [Na^+] (Murphy *et al.* 1986) and to increase tibial length (Thommes *et al.* 1992). Thus, while exogenous GH may not be able to augment normal growth rate during embryonic development, it appears to have numerous actions not directly related to whole body growth.

The presence of GHRs in the early chicken embryo may also account for the subcellular distribution of GH in many embryonic tissues. Although GH might be expected to be present in the cytoplasm of GH-producing cells, GH immunoreactivity

was often associated with the nucleus (eg. Fig 2.8). It is, however, well established that following its binding to membrane receptors GH is rapidly internalised to nuclear compartments in rat target cells (Fraser and Harvey, 1990). Nuclear and peri-nuclear GH has therefore frequently been detected in other immunocytochemical studies (Rezvani *et al.* 1973; Bonifacino *et al.* 1983). A nuclear localisation of GH produced within GH-producing cells may also occur if it escapes packing in the Golgi and this is particularly likely in rapidly growing or differentiating embryonic cells, as demonstrated in proliferating or tumurous mammary tissues (Mol *et al.* 1996).

The internalisation of GH in its target sites has been shown to provide an extra-pituitary store of GH in peripheral tissues. The amount of GH in extra-pituitary tissues is, however, minimal in comparison with that in the pituitary somatotrophs. For instance, GH immunoreactivity in the chicken brain, thymus, and bursa accounts for <1% of that in the pituitary gland (Render *et al.* 1995a,b). The pituitary is also primarily responsible for plasma GH, since circulatory GH levels are not detectable following pituitary ablation in chicken and turkeys (Harvey and Scanes, 1977; Leung *et al.* 1986). Extra-pituitary tissues that produce GH are therefore unlikely to be GH-secreting endocrine glands. The storage of GH in the pituitary gland thus appears to be associated with peri- and postnatal development. During embryogenesis, GH may thus be a paracrine or autocrine rather than an endocrine regulator of growth. These roles may, furthermore, reflect ancestral roles of GH, since extra-pituitary GH-like proteins are present in primitive vertebrates lacking pituitary glands (Wright, 1986)

and in the neural ganglia of invertebrates (Swinnen *et al.* 1990; Inestrosa *et al.* 1990).

This study investigated the expression of GH and GHR during early chick embryogenesis. An earlier study (Wang, 1989) demonstrated that GH immunoreactivity is present on ectodermal and endodermal layers of the ED 1 and 2 chick embryo, and on the reproductive tracts of the early chick embryo. However, the results of the present study show that GH is present in all tissues of the early embryo before the release of GH by pituitary somatotrophs (Porter, 1997). This is the first study to show the expression of GH and GHR in the same tissues of the early avian embryo. This work supports the recent discovery of GH and GHR in early murine embryos (Panteleon *et al.* 1996), suggesting that the presence of extra-pituitary GH-producing cells in vertebrate embryos are the rule rather than the exception. Furthermore, the recent detection of immunoreactive luteinising hormone (LH) in the stomach and lung bud of early chick embyros (Shirasawa *et al.* 1996) indicates that pituitary hormones may be expressed in extra-pituitary tissues of the chick embryo before their expression in the pituitary gland. The presence of GH and GHR within embryonic tissues suggests that these may be sites of autocrine or paracrine GH action.

Table 2.1. GH and GHR-immunoreactivity in tissues of early chick embyros.

	Age (embryonic day)				
Tissue	3	5	6	7	8
epidermis	+++	+++	+++	+++	ND
neural tube	+++	ND	+++	+++	ND
notochord	+++	ND	+	+	ND
mesoderm	+++	+++	+++	+++	ND
limb bud mesoderm	NP	++	+++	ND	ND
cartilage	NP	ND	ND	ND	+++
Somites:					
myotome	+++	ND	+++	+++	ND
scleretome	+++	ND	+	+	ND
dermatome	+++	ND	+	+	ND
neural retina		ND	+++	+++	ND
nerve ganglia	+++	ND	+++	+++	ND
Lens:					
cortical cell nuclei			+++	ND	ND
central cell nuclei			+++	ND	ND
equatorial cell nuclei			+++	ND	ND
heart	+++	ND	ND	ND	ND
stomach	+++	ND	ND	ND	ND
Kidney:					
mesonephros	+++	++	++	ND	ND
Wolffian duct		+++	ND	ND	ND
Amnion:					
ectodermal layer	+++	ND	+++	+++	ND
Mesodermal syncitium:					
cytoplasm	-	-	-	-	ND
nuclei	NP	ND	+++	ND	ND

Abbreviations: +/- = scattered immunoreactive cells within a tissue; +++ = intense immunoreactivity; - = no immunoreactivity; ND, not determined.

Fig 2.1. GH-immunoreactivity in the adult chicken anterior pituitary gland. This sagittal section iluustrates the distribution of immunoreactive somatotrophs in the caudal lobe (Ca) using αGH1 (Harvey *et al.* 1979). Isolated cells and small groups (clumps) are also found in the cephalic lobe (Ce) (arrows). Bar = 1mm. Staining was obtained using the ABC technique.

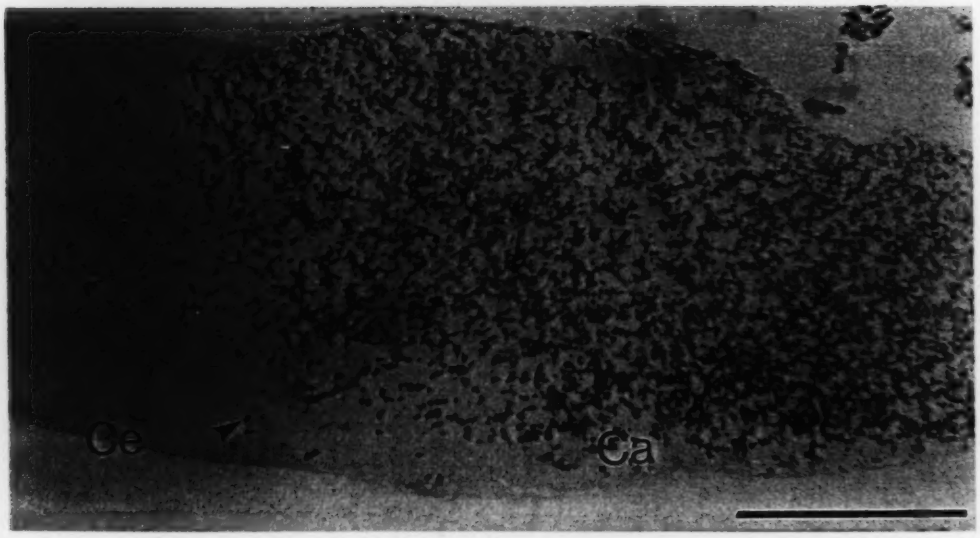
Ce
Ca

Fig 2.2 GH-immunoreactivity in the ED 3 embryo. This figure shows the widespread distribution of GH-immunoreactivity in the ED 3 embryo. GH-immunoreactive cells were detected using 2 polyclonal antibodies (αGH1 and αGH2) raised against cGH and a mouse monoclonal antibody (αIH7) raised against glycosylated cGH. These cells were visualised by a biotinylated antibody, avidin-peroxidase complex and diaminobenzidine (DAB) (Fig 2.2 E; F) or DAB + nickel ions which produces a black substrate (Fig 2.2 C; D). The brown colouration of Fig 2.1 A was achieved using a secondary antibody conjugated to alkaline phosphatase, APAAP and Fast Red TR/Napthol AS-MX colour substrate. A: Transverse section through mesonephros using αGH1 (Harvey *et al.* 1979). B: Preabsorption of polyclonal αGH2 with recombinant cGH abolished staining in an adjacent section to A. C: Transverse section through the level of the mesonephros and dorsal aorta using αGH2 (Porter *et al.* 1995a). D. Preabsorption of the αGH2 with recombinant cGH abolished staining in a similar section to C. E: Transverse section through level of stomach stained with mouse monoclonal αIH7 (Berghman *et al.* 1992). F: Preabsorption of αIH7 with recombinant cGH abolished staining. Abbreviations: a: amnion; at: atrium; e: epidermis; en: endoderm: mesonephros; n: notochord; nt: neural tube; pcv: posterior cardinal vein; s: somite; st: stomach; m: mesencephalon; md: mesonephric duct; so: somatopleure; sp: splanchnopleure. Bar = A, B, C and D: 80μm; E and F; 100μm.

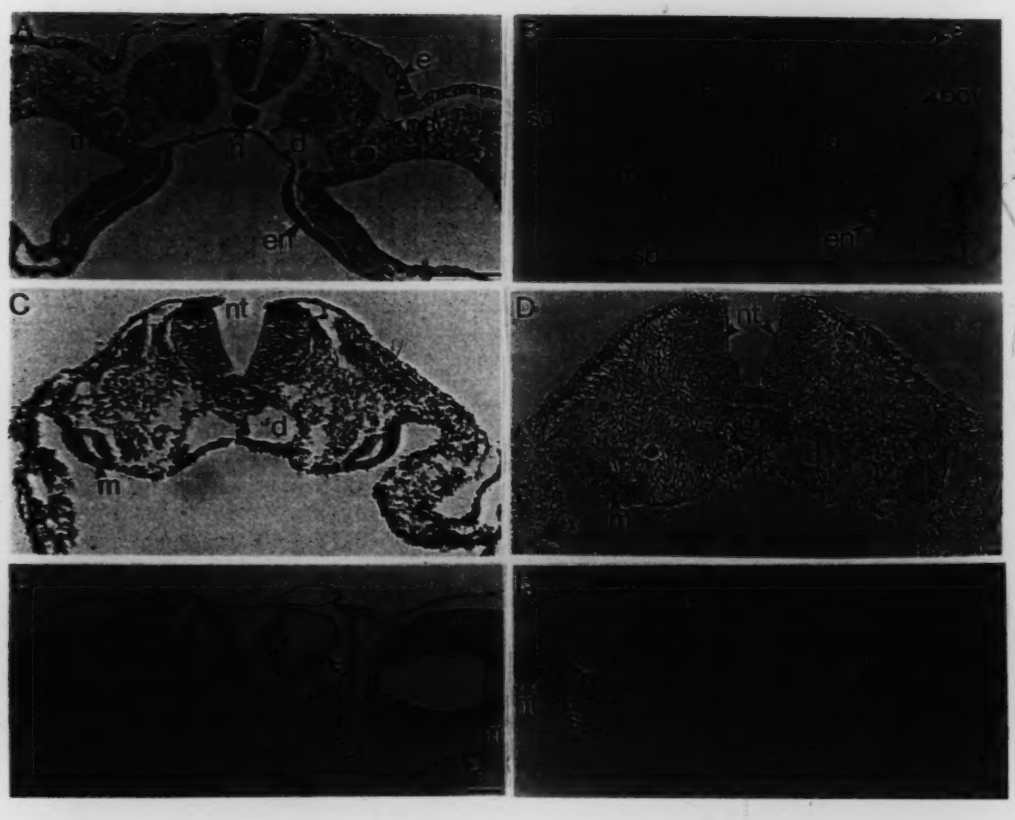
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Fig 2.3. GH-immunoreactivity in the ED 5 Wolffian duct. A: Transverse section through Wolffian duct (Wd). Staining is present in some cells of the duct (arrows). Lighter staining is also seen in scattered cells of the kidney tubules (tu). B: Preabsorption of αGH1 with recombinant cGH completely abolished staining. C: Higher power of A showing the strong staining in the Wolffian duct. D: High power magnification of the Wolffian duct. Note that not all cells of the Wolffian duct are immunoreactive for GH. Bar = 100μm. Abbreviations: e, epidermis; m, mesoderm. The ABC technique of staining was used and αGH1 as the primary antibody.

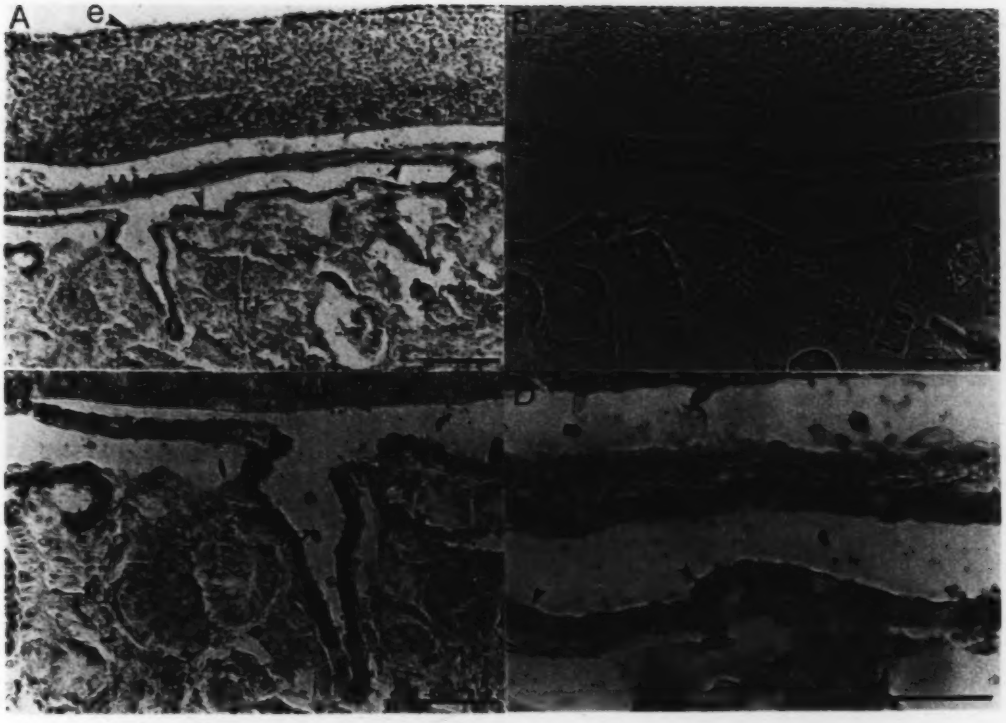
A
e

Fig 2.4. GH-immunoreactivity in the ED 6 embryo. A: Transverse section at level of the wing buds. GH-immunoreactivity was widespread throughout the embryo although the notochord (n) appeared to have lost its immunoreactivity. Strong immunoreactivity was seen in the amnion (a), myotome (my), neural tube (nt), limb bud mesenchyme (L), mesonephric tubules (me) and throughout the mesoderm (m) of the body. B: Preabsorption of the αIH7 with recombinant cGH abolished all staining. C: High power magnification of the mesoderm from section A. Many cells show dense staining for GH although there are cells (arrows) which are not stained. D: Preincubation of the αIH7 with recombinant cGH did not result in staining. Abbreviations: pcv, posterior cardinal vein; d, dorsal aorta. Staining was obtained using the ABC technique and αIH7 as the primary antibody. Bar = 250μm (A and B), or 20μm (C and D).

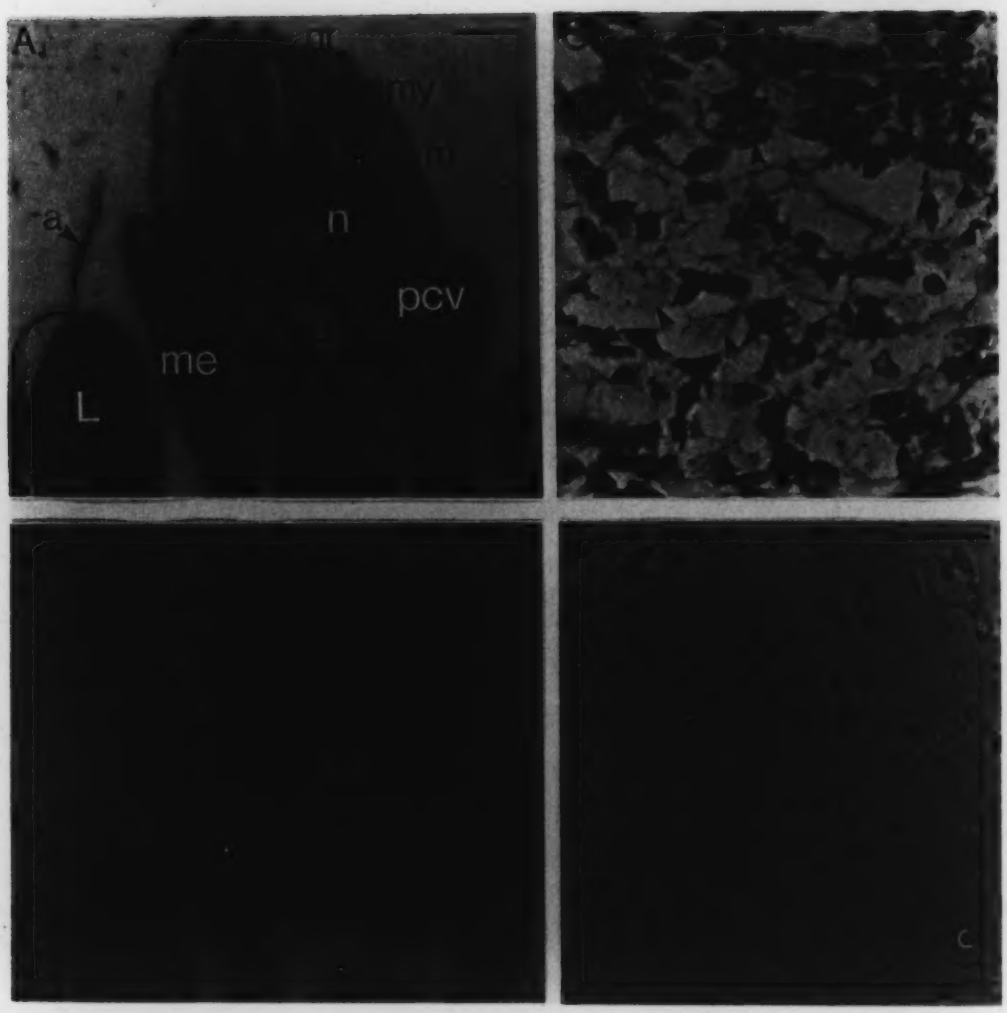
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Fig 2.5. GH-immunoreactivity in the ED 6 extra-embryonic membrane. A: High power magnification of the amnion taken from a transverse section of a 6 day embryo stained with the αIH7. The ectodermal layers (ec) of the amnion are strongly immunoreactive. B: Preabsorption of the αIH7 with cGH reduced staining. C: The mesodermal (m) layer of the amnion is beginning to differentiate from a syncitium into musculature and the nuclei (n) of these cells are very intensely GH-immunoreactive. Bar = 50μm. D: Preabsorption of the αIH7 with recombinant cGH reduced nuclear staining. The ABC method was used and αIH7 was the primary antibody.

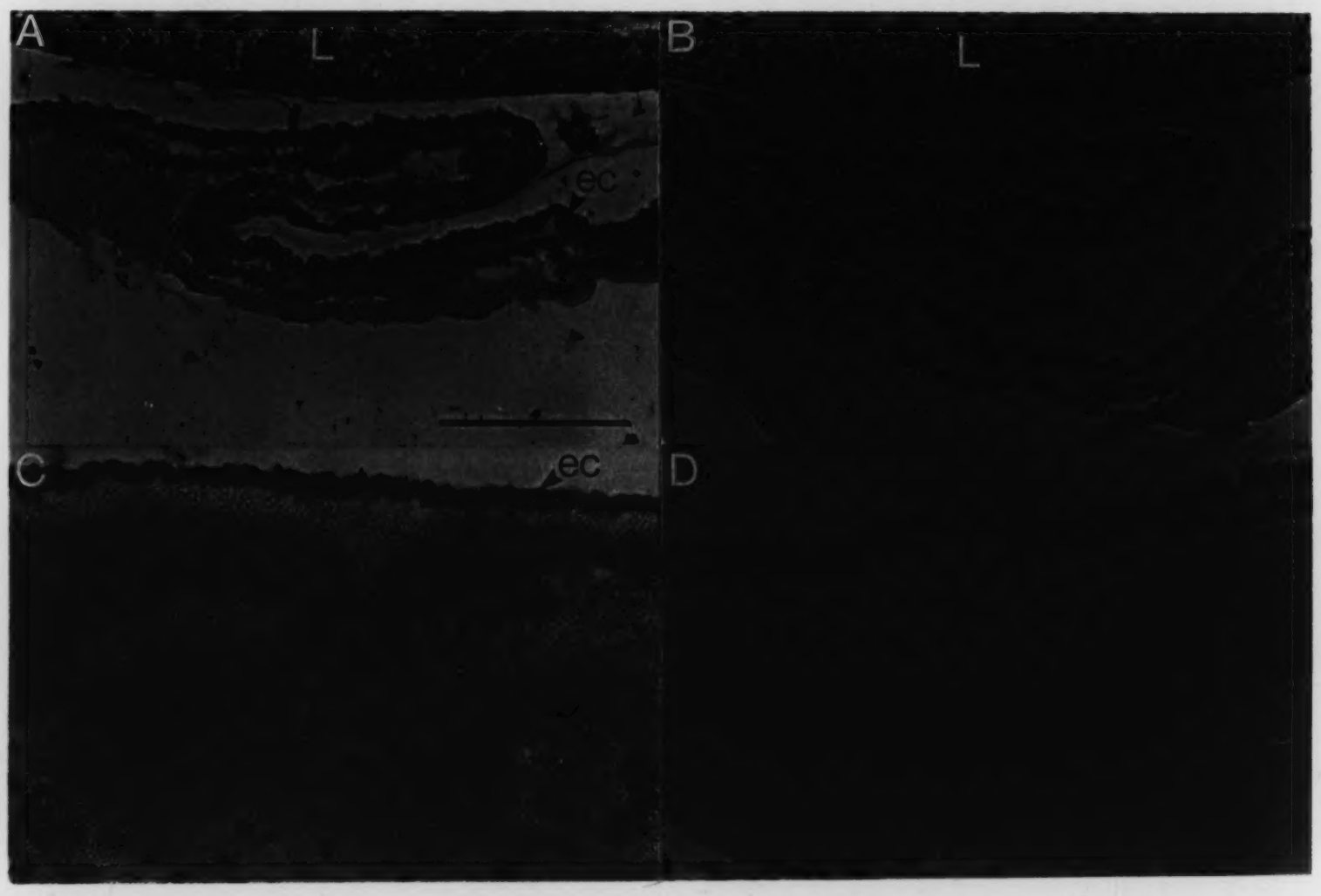
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Fig 2.6. GH-immunoreactivity in the ED 7 embryo. A: The amnion of the 7 day embryo was intensely GH-immunoreactive. Bar = 20μm. B: The epidermis (ep) and myotome (s) were also immunoreactive, although the mesederm (m) had appeared to have lost the majority of its immunoreactivity seen in the ED 6 embryo. Immunoreactivity was restricted to certain cells in the mesoderm scattered within this tissue (Fig 2.6 C). Bar = 50μm. C: The endothelium (en) of a blood vessel showed intense GH-immunoreactivity. Bar = 10μm. D: Preabsorption of αGH1 completely abolished staining in a section similar to C. Bar = 10μm. Abbreviations: b: blood cells; ep: epidermis; m: myotome; me: mesoderm; nt: neural tube Staining was achieved using the APAAP method and αGH1 as the primary antibody.

ep
mr

B
ep

Fig 2.7. GH and GHR-immunoreactivity in the ED 8 limb bud. A: GH-immunoreactivity in cells from the hypertrophic zone stained with αGH1. Some cells show strong cytoplasmic staining (c) others show strong nuclear staining (n) and others do not appear to be immunoreactive (arrows). B: GH-immunoreactivity in the cytoplasm of cells undergoing mesenchymal condensation from the zone of flattened cells. C: High magnification of GHR-immunoreactivity in cells from the hypertrophic zone. Notice the strong nuclear staining and less dense cytoplasmic staining. Some cells do not appear to be immunoreactive (arrows). D: Preincubation of αGH1 with recombinant cGH completely abolished staining in cartilage cells. An adjacent section to A is shown for illustration. Bar = 10μm. Staining was achieved using the ABC method and αGH1 was the primary antibody.

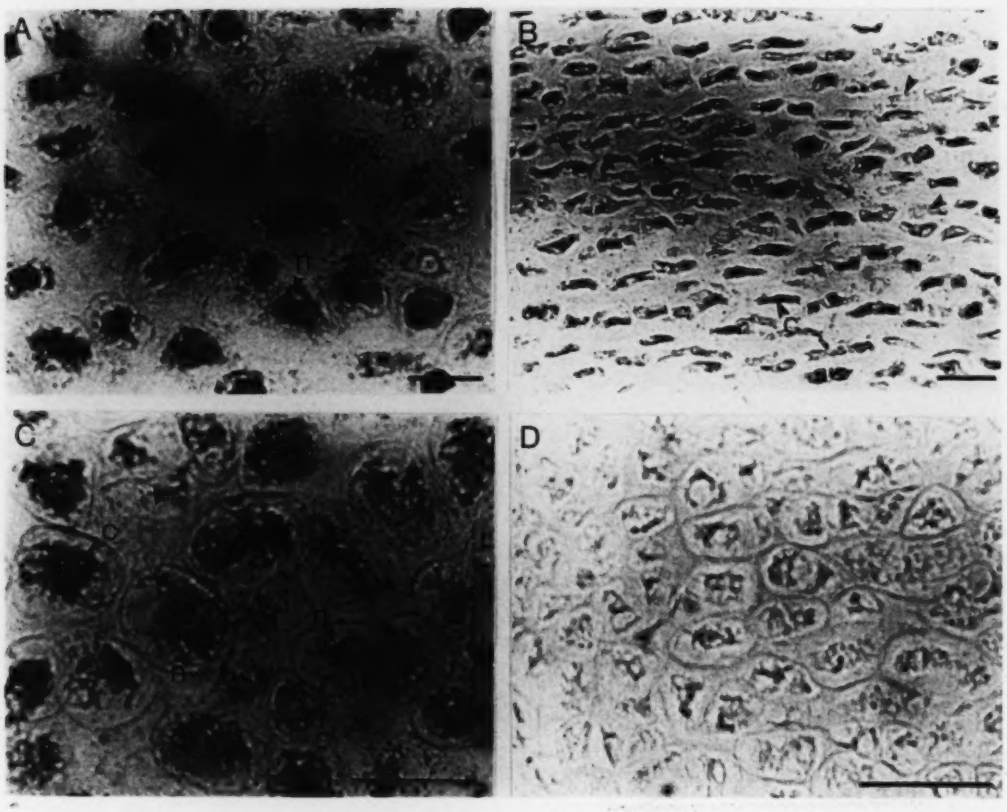
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Fig 2.8. GHR-immunoreactivity in the ED 3 embryo. Transverse section through the mesonephric duct stained for GHR. The widespread immunoreactivity is similar to the distribution of GH-immunoreactivity seen in Fig 2.1. A. Bar = 80μm. Staining was not observed when the antibody was replaced with non-immune rabbit serum or PBS (data not shown). Abbreviations: e: epidermis; en: endoderm; c: coelom; d: dorsal aorta; n: notochord; nt: neural tube; s: somite; so: somatopleure; sp: splanchnopleure.

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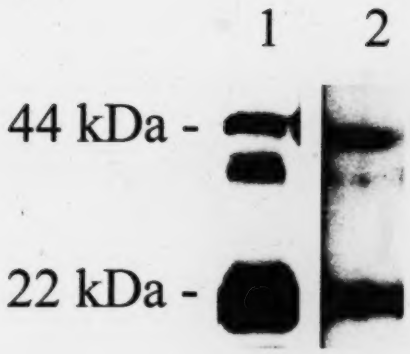

Fig 2.9. Western analysis of proteins from the headless body of the ED 7 embryo. Growth hormone (GH)-like immunoreactivity was detected by an antibody raised against native pituitary chicken growth hormone (Harvey and Scanes, 1977). GH-like proteins of appropriate size were present in the adult pituitary (1) and ED 7 embryo (2). The 22 kDa corresponds to the monomer form of chicken GH and the 44 kDA probably corresponds to a GH dimer. Protein extracts were derived from a pool of 5-10 embryos.

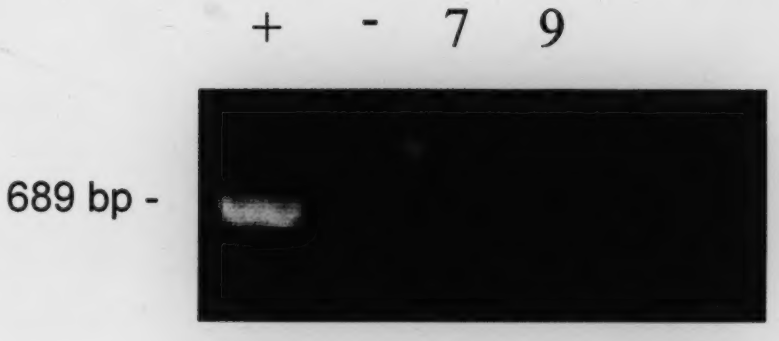

Fig 2.10. Analysis of ED 7 and ED 9 GH transcripts by the polymerase chain reaction (PCR) in the presence of GH3 and GH5 primers. RNA from adult pituitary (+), ED 7 headless bodies (7) and ED 9 headless bodies (9) was reverse transcribed and amplified in the presence of the primers (GH 3' and 5'). The amplified cDNA was visualised by electrophoresis in ethidium-bromide-stained 1.2% minigels. The second lane (-) shows adult pituitary RNA in the absence of reverse transcriptase. RNA was obtained from a pool of 5-10 embryos.

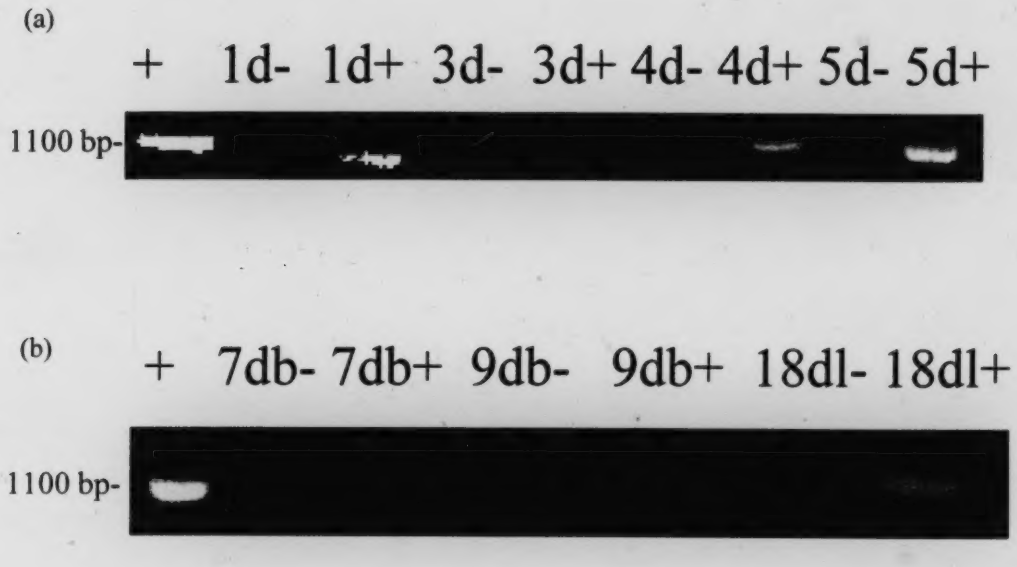

Fig 2.11. Ontogeny of growth hormone receptor (GHR) gene expression in chick embryos. Total RNA was reverse transcribed and amplified by 30 cycles of polymerase chain reaction (PCR). (a) PCR of RNA from the headless bodies of 1, 3, 4 and 5 day old embryos. (b) PCR of RNA from the headless bodies of 7 and 9 day embryos and from livers of 18 day old embryos (18dl). RNA was reverse transcribed in absence (-) or presence (+) of reverse transcriptase and RNA from liver tissue acted as controls. Protein homogenates were obtained from a pool of at least 10 embyros.

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Chapter 3

Growth Hormone in Neural Tissues of the Chick Embryo

Some of the results in this chapter were presented at the 6th International Symposium on Avian Endocrinology (1996), Lake Louise, Canada (Abstract 3.12) and the 5th Western Perinatal Research Meeting (1997), Banff, Canada (Abstract 8.12). Data from this chapter were also published in C.D.M.Johnson, M.A. Wride, K.L. Hull, and S. Harvey (1996). Immunohistochemical Localization of Growth Hormone and Growth Hormone Receptor in the Early Chick Embryo. *Poultry and Avian Biology Reviews*, Vol 6, 4: 259, and S. Harvey, C.D.M. Johnson, P. Sharma, E.J. Sanders and K.L. Hull (1998). Growth Hormone: An Embryonic Paracrine? *Comparative Biochemistry and Physiology,* (in press).

I. INTRODUCTION

Most anterior pituitary hormones are present within the central nervous system (CNS) (Kreiger, 1980, 1983) including growth hormone (GH) (see Harvey *et al.* 1993 for review). GH has been identified in fish, avian and mammalian brains and is expressed in all major areas of the CNS. In the adult chicken brain, immunoreactive GH-like proteins have been detected by radioimmunoassay (RIA) and immunoblotting in the hypothalamus and in extra-hypothalamic tissues (Render *et al.* 1995). These proteins were similar in size and antigenicity to pituitary GH.

The presence of GH in the brain of vertebrates may partly reflect the sequestration of pituitary GH. This would involve its passage across the blood-brain-barrier (BBB), or retrograde flow through the hypophyseal portal blood vessels (Sato *et al.* 1989). It is, however, generally considered that GH is unable to cross the BBB (Beltchez *et al.* 1982) and GH concentrations in systemic circulation are poorly correlated with GH concentrations in cerebrospinal fluid (CSF) (Doering and Chang, 1991). The presence of GH within the brain is thus likely to reflect transcription of the GH gene or a GH-like gene in neural tissues. Indeed, it is now well established that the brain is an extra-pituitary site of GH production. In chickens, RT-PCR of mRNA from brain tissue generated cDNA identical in size to pituitary GH cDNA, indicating the transcription of the pituitary GH gene in neural tissues. The presence of GH mRNA in many areas of the rat and human brain further indicates that the brain is a site of extra-pituitary GH synthesis (Gossard *et al.* 1987; Martinoli *et al.* 1991).

Similarly, the persistence of GH in the brains of rats after hypophysectomy also suggests synthesis of GH within the CNS (Pacold *et al.* 1978; Hojvat *et al.* 1982a; Hojvat *et al.* 1982b). Furthermore, immunoreactive GH has been detected immunocytochemically in neuronal perikarya, processes and boutons, suggesting a neuronal site of synthesis (Lechan *et al.* 1981; Lechan *et al.* 1983).

However, very little is known regarding the ontogeny of brain GH, since it has primarily been detected previously in adult mammals, or postnatal birds (Render *et al.* 1995a). However, the detection of immunoreactive GH in the brain of foetal rats (on the 10th day of gestation) prior to the appearance of pituitary GH (on day 12), indicates that it is produced perinatally, and surprisingly, appears to have an earlier ontogeny than pituitary GH (Hojvat *et al.* 1982).

The embryo has been generally considered GH resistant, and the embryonic growth period is thought to be controlled by peptide growth factors (e.g., fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factors (IGF-I and II) and unidentified serum growth factors (see Geffner, 1996 for review). However, it has been recently shown that several embryonic/foetal tissues respond to GH (Stracke *et al.* 1984; Strain *et al.* 1987; Slootweg *et al.* 1988). For instance, exogenous GH can restore growth in embryonic rats after transplantation of parts of embryos into hypophysectomised hosts (Nicoll *et al.* 1991). Moreover, the growth hormone receptor that is expressed throughout the CNS of postnatal vertebrates (see Harvey *et al.* 1993 for review) has also been identified in the CNS of foetal rats

(Garcia-Aragon *et al.* 1992; Hasegawa *et al.* 1993) and foetal humans (Hill *et al.* 1992). It is therefore possible that brain GH may have autocrine or paracrine roles in regulating neural function or neuronal growth during early ontogeny. The demonstration that GH is essential for normal brain development (Laron *et al.* 1985) and for neuronal differentiation (Pelton *et al.* 1977) supports this view. In the present study the ontogeny and distribution of GH in neural tissues has therefore been further evaluated in embryonic chicks.

II. MATERIAL AND METHODS

Tissues

Tissues of chick embryos were prepared and processed as in Chapter 2. The morphological stages of development during incubation were based on Hamburger and Hamiliton, (1951). Neural structures were identified according to Romanoff (1960), Patten (1971), Kuenzel and van Tienhoven (1982) and Uryu *et al.* (1988).

Immunocytochemistry

Immunocytochemistry was performed on selected neural tissues of the ED 3, 6, 7, 14 and 19 embryo and on the of eye of the ED 6, 7 and 14 embryo. Staining was carried out as outlined in Chapter 2.

Western analysis

Selected neural or eye tissue (from at least 5 embryos) were subected to Western analysis as outlined in Chapter 2.

Polymerase chain reaction *(carried out by P. Sharma)*

The presence of GH mRNA in selected neural and eye tissue was assessed using the polymerase chain reaction (RT-PCR) as detailed in Chapter 2.

III. RESULTS

Immunocytochemistry

The distribution of GH-immunoreactivity in the neural tissues of chick embryos are summarised in Table 3.1.

At ED 3 the neural tube develops anteriorally to give rise to the brain. The principle divisions of the brain, in birds, from anterior to posterior are the telencephalon, (which develops into the cerebral hemispheres), the diencephalon or thalamus, the mesencephalon or mid-brain, the metencephalon or cerebellum and the myelencephahlon or medulla oblongata. At ED 3 all these divisions are present and the lumen of each division is continous with the others. In other words, the brain is still a tube and its inner walls consist of developing brain tissue.

At ED 3 all major divisions of the brain were GH-immunoreactive. Fig 3.1A shows that the telencephalon is strongly immunoreactive. The specificity of this staining is indicated by its abolition after the primary antisera had been preincubated with recombinant cGH (Fig 3.1B). The replacement of the antisera with NRS similarly failed to stain adjacent sections (data not shown). The otic vesicle, which develops into the ear was also strongly GH-immunoreactive (Fig 3.1C). Several cranial ganglia were also GH-immunoreactive. The Vth (semi-lunar) nerve, VIIth (facial) nerve, VIIIth (acoustic) nerve, and IXth (glossopharyngeal) nerve ganglia were strongly GH-immunoreactive (Fig 3.1C, 3.1E). (The VIIth and VIIIth nerve develop in close association and it is not possible to distinguish them apart at 3 days on incubation. These are therefore referred to as the facial-acoustic nerve. This immunoreactivity was lost following preabsorption of the primary antisera with recombinant cGH (Fig 3.1D). The neural tube, posteriorally, was also GH-immunoreactive, with a similar staining intensity as the anterior neural tube (Fig 3.1F).

The widespread distribution of GH-immunoreactivity in the neural tissues of the ED 3 embryos were mirrored by the distribution of GHR-immunoreactivity (Fig 3.2). GHR-immunoreactivity was similarly ubiquitous in the brain divisions. The mesencephalon (Fig 3.2A) is characteristic of the staining of other brain regions. Strong GHR staining was seen in the wall of the mesencephalon and the surrounding meseodermal tissue. The optic vesicle, which develops into the eye, was strongly

GHR-immunoreactive (Fig 3.2C), the otic vesicle was GHR-immunoreactive (Fig 3.2B) and several cranial nerve ganglia were staining for GHR (Fig 3.2B, 3.2D). The Vth, VII/VIIIth, and IXth nerve ganglia showed intense GHR-immunoreactivity. The surrounding mesoderm tissues were also GHR-immunoreactive.

The optic cup and lens of ED 6 embryos stained for GH (Fig 3.3A). The lens fibre nuclei cells were shown to be intensely GH-immunoreactive (Fig 3.3C). This was ontogenically the first indication that the nuclei of these cells were immunoreactive. The cytoplasm of the lens fibre nuclei cells also stained, although less strongly than the nuclei (Fig 3.3C).

By 7 days of incubation (stage 31) GH-immunoreactivity was still abundantly present in the optic cup (Fig 3.4). The neural retina was intensely stained for GH (Fig 3.4B), although the mesodermal layer (m) lying above the pigmented retina (pr) was much less immunoreactive, with only a few cells (arrows) staining for GH. The epidermis of the head is visible on plate 3.4B and was also GH-immunoreactive.

The diencephalon and Rathke's Pouch of the ED 7 embyro were strongly stained for GH-like proteins (Fig 3.5A). The otic vesicle also showed strong immunoreactivity in the 7 day embryo (Fig 3.5B). The cells in the ganglia associated with the developing ear were GH-immunoreactive, as were the facial/acoustic ganglia (VII and VIII nerves) (Fig 3.5 B) and the V nerves (Fig 3.5 C).

The lens of the ED 14 embryo (stage 14) showed a similar distribution of GH-immunoreactivity to that seen in the lens of the ED 6 embryos. The nuclei of the lens

fibre cells, equatorial epithelia cells, cortical fibre cells and the nuclear fibre cells were densely stained for GH (Fig 3.6A). However, GHR-immunoreactivity was shown to be present in the cytoplasm throughout the lens and was not restricted to the nuclei (Fig 3.6C).

The brain of 14 day old embryos (stage 14) was still very GH-immunoreactive (Fig 3.7). The choroid plexus (circumventricular organ) stained particularly strongly for GH (Fig 3.7 A). This staining was lost when the primary antibody was preabsorbed with recombinant cGH (Fig 3.7 B). Within the cerebral cortex, many cells had GH-immunoreactivity within the molecular and pyramidal layer.(Fig 3.7 C).

Within the cerebral cortex of ED 14 embryos many cells were GH-immunoreactive. Fig 3.8A shows neuroglial cells from the gray matter which are probably astrocytes due to their position and morphology. Numerous smaller cells were also strongly stained for GH (Fig 3.8B). This staining was lost following the preabsorption of the primary antibody with recombinant cGH (Fig 3.8F). Large pyramidal neurons were intensely stained for GH (Fig 3.8C). Large cells from the pyramidal neuron layer showed very dense nuclear GH-immunoreactivity (Fig 3.8D). Nerve tract fibres located in the white matter of the cerebral cortex did, however, also contain GH-immunoreactivy (Fig 3.8E).

Within the cerebellum of the ED 14 embryo, the cells of the gray matter were intensely stained for GH (Fig 3.9A and B). The Purkinje cells which are located between the gray and molecular layers of the cerebellum were also strongly GH-

immunoreactive (Fig 3.9B). The molecular layer and the white matter did not appear to be as strongly stained (Fig 3.9A). Preincubation of the primary antibody with recombinant cGH completely abolished staining in the cerebellum (Fig 3.9C).

At the 19th day in incubation, (stage 45) the subtrochlear organ was intensely stained for GH (Fig 3.10A). The fasciculus longitudinalis medialis (fibre tract) also stained, although less intensely, than the subtrochlear organ (Fig 3.10A and B). The preincubation of the primary GH antibody resulted in a complete loss of staining (Fig 3.10C).

Western analysis

GH-like proteins of approximately 22kDa and 44kDa were present in the head (with and without the pituitary gland) (Lane 3 and 4, Fig 3.12), and in the eye (Lane 2, Fig 3.11). These GH-like proteins correspond to GH moieties present in the chicken pituitary gland (Lane 1, Fig 3.11).

Polymerase Chain Reaction

In the presence of oligonucleotide primers for pituitary cGH cDNA (GH_3 and GH_5), a cDNA moiety of approximately 689 bp was amplified from the head of ED 7 and ED 9 embryos (Fig 3.12). Adult pituitary RNA that had been reverse transcribed in the presence or absence of reverse transcriptase acted as positive and negative

controls respectively. This moiety was identical in size to the cDNA generated from adult pituitary RNA.

IV. DISCUSSION

This is the first study to reveal the presence of GH-like proteins within the developing brain and other neural tissues of the chicken embryo. The presence of GHR-immunoreactivity within these areas suggests that GH possesses biological activity within neural tissues of the early embryo. This work also raises questions regarding the origin and the evolutionary history of neural GH.

The results of the present study are in accordance with the established presence of GH and GHR within the adult mouse, rat, chicken and primate CNS (reviewed by Harvey *et al.* 1993) and it supports the studies that have detected GH in rat (Hojvat *et al.* 1982) neural tissues.

Growth hormone-immunoreactivity was found to be ubiquitously present in the developing brain of ED 3, 4, 6 and 7 embryos and to be present in discrete brain regions of 14 day and 19 day chick embryos. The presence of GH in the embryonic brain, before the ontogenic appearance of GH-releasing somatotrophs (16 days) (Porter *et al.* 1995a), plasma GH (17 days) (Harvey and Scanes, 1977), and pituitary GH mRNA (18 days) (McCann-Levorse *et al.* 1993) is likely to reflect an earlier transcription and translation of the GH gene in these neural tissues. Indeed, in foetal rats, brain GH was similarly detected on day 10 of incubation, two days before the

appearance of pituitary GH (Hojvat *et al.* 1982a). Therefore, it is unlikely that neural GH is sequestered from pituitary GH.

Reports on the molecular size of monomeric cGH have ranged from 22 to 26 kDa (Harvey and Scanes, 1977; Lai *et al.* 1984; Souza *et al.* 1984; Houston and Goddard, 1988), depending on the experimental analysis. The detection of a GH-like protein of approximately 22 kDa in neural tissues of the chick embryo corresponds to the size of monomeric pituitary cGH, suggesting they are immunologically similar. Furthermore, the identification of GH-like mRNA in the 7 day brain, suggests the transcription of the pituitary GH gene within these tissues. It has been shown previously that brain GH in adult chickens is identical to pituitary GH. The GH-like protein in the rat brain is also immunologically similar to pituitary GH (Hojvat *et al.* 1982; Lechan *et al.* 1983), although it does resemble the mid-portion of the human GH (hGH) more closely than the native rat GH (rGH), suggesting that rat brain GH may not be identical to pituitary GH. The present study also demonstrated the existence of a 44 kDa GH moiety in the 7 day brain and it is possible that this reflects a GH dimer.

The widespread detection of GH- and GHR-immunoreactivity in neural tissues of the chick embryo, raises the possibility that GH may posses roles in the development and function of the CNS. GH-immunoreactivity was found in the perikarya of pyramidal neurones within the cerebral cortex and in the Purkinje cells of the cerebellum of 14 day embryos, suggesting neural rather than glial sites of

synthesis. GH-like proteins have previously been detected in neuronal perikarya, processes and terminal boutons in rat brain (Lechan *et al.* 1981; Lechan *et al.* 1983) and the increase in immunoreactive GH staining after colchicine blockade of axonal transport (Lechan *et al.* 1981), supports this view .

The presence of GH- and GHR-immunoreactivity within the brain and nerve ganglia of the chick embryo raises the possibility that GH has neurotrophic roles in the chick embryo. The cranial nerves, which are derived from the cephalic portion of the neural crest, undergo extensive growth and differentiation from 3 days onwards. In GH-deficient states, either induced artificially or in clinical pathologies, severe deficits in brain growth and development are observed. For instance, in immunologically induced GH-deficient rats, myelination of axons is markedly impaired (Pelton *et al.* 1977) and RNA/DNA synthesis in these cells is also reduced. GH-deficient mice similarly have a microcephalic cerebrum, with hypomyelination, retarded neuronal growth, poor synaptogenesis and behavioural abnormalities (Noguchi, 1996). The hypomyelination in these mice is due to reduced glial cell proliferation, which suggests that GH action on these cells is a necessary precondition of myelination.

GH has been shown to stimulate neuronal and glial cell proliferation, as shown by increased ornithine decarboxylase (ODC) activity (a marker for myelination) in neonatal rats (Roger *et al.* 1974). GH also directly stimulated RNA synthesis in rat cerebral slices in vitro (Krawiec and Berti-Mattera, 1985). In the

present study, very intense GH-immunoreactivity was detected in pyramidal cells of the cerebral cortex, and it is possible that GH is influencing their development. Indeed, exogenous GH administration to pregnant rats resulted in an increase in the number and length of cortical pyramidal cell dendrites (Zamenhof *et al.* 1966).

The presence of GH-immunoreactivity within nerve fibres of the brain of ED 14 embryos suggests a neuromodulatory role for GH. Indeed, GH-immunoreactivity has been demonstrated in rat nerve fibres in association with SRIF and TRH-neurones (Lechan *et al.* 1981; Lechan *et al.* 1983). The presence of GHR mRNA in GRF- and SRIF-neurones of rabbit and rat hypothalamus (Fraser *et al.* 1990), may therefore suggest an autoregulatory role for GH in regulating pituitary GH secretion. GH tracts have also been identified in the brains of fish (Hansen and Hansen, 1982) and immature sea lampreys (Wright *et al.* 1986). In postnatal chickens, exogenous GH reduces the content and turnover of dopamine (DA) and noradrenaline in the hypothalamus (Lea and Harvey, 1993). Endogenous brain GH may therefore have negative feedback effects upon pituitary GH. As GH and GHRs are present within the brain it is also possible that endogenous GH may have neuroendocrine and neuromodulatory roles.

The presence of GH-immunoreactivity in the 14 day chick choroid plexus is in agreement with previous studies which have detected GH (Hojvat *et al.* 1983; Lechan *et al.* 1983) and GH mRNA (Garcia-Aragon *et al.* 1992) in the choroid plexus of rats. It is possible that production of GH by this circumventricular organ is

secreted into the cerebrospinal fluid (CSF). However, this could also reflect receptor bound GH which allows its transport through the BBB.

The present study also detected GH-immunoreactivity in the subtrochlear organ (STO) and associated fasiculus longitudinalis medialis of the 19 day embryo. This circumventricular organ is found in the posterior mesencephalon of the chicken and is an elaborate structure with modified ependymal cells (Kuenzel and van Tienhoven, 1982). It is believed to form from the embryonic sulcus intraencephalicus (Uryu *et al.* 1988). This structure occurs in the same area of the pontine flexure where dynamic changes in the angle of the brain with respect to the spinal cord occur (Schumacher 1928; Romanoff 1960; Uryu *et al.* 1988). It is not known when the sulcus intraencephalicus is transformed into the STO, although its demonstration in this study would suggest that it had formed by 19 days of embryonic development. Although, the physiological function of the STO is unknown, the fine structure of this organ has been investigated (Uryu *et al.* 1988). Ependymal cells lining the STO have irregular apical surfaces which are characteristic of cells designed for absorption and/or secretion (Flament-Durand and Brion, 1985), such as ependymal cells which line the ventricles. Therefore, the presence of immunoreactive GH in these ependymal cells could suggest the secretion of GH by these into the CSF or the absorption of GH from the CSF.

The demonstration of GH- and GHR-immunoreactivity within the developing eye in the chick embryo suggests that GH has roles affecting the development and

growth of this sensory organ. There are many polypeptide growth factors and cytokines which affect lens differentiation (Piatigorsky, 1981; Tripathi *et al.* 1991; Wride, 1996) and it is possible that GH is one of them. It is interesting that IGF-1 is located in the neural retina of chicks and it is therefore possible that GH may be acting via this peptide. Furthermore, somatostatin like immunoreactivity was detected in the retinae of embryonic and adult chickens (Ellis *et al.* 1983), indictating that its production or release may be modified by the same releasing factors that regulate pituitary GH secretion.

Finally, GH- and GHR-immunoreactivity was observed in the developing ear. The 3 day otic vesicle stained intensely and uniformly for GH and GHR and at 7 days the otic vesicle was still GH-immunoreactive. The otic vesicle develops from a thickened area of ectoderm, first identifiable in 36 hour embryos. This is the first demonstration of GH in a progenitor of the auditory organ.

In summary, the results of this study clearly establish that neural tissues of the chick embryo express GH and GHR during early embryogenesis, before the development of the pituitary gland and the release of GH by pituitary somatotrophs (Porter, 1997). This would suggest that GH may have autocrine or paracrine functions within neural tissues and possess important roles in neural tissue growth and development.

Table 3.1 Growth hormone (GH)- and GH receptor-immunoreactivity within neural tissues of the chick embryo

AGE	LOCATION	GH	GHR
ED 3	neural tube	+++	+++
"	optic vesicle	+++	+++
"	otic vesicle	+++	+++
"	cranial nerves	+++	+++
"	spinal ganglia	-	-
ED 4	neural tube	+++	ND
"	spinal ganglia	++	"
ED 6	neural tube	+++	"
"	optic cup	+++	"
"	lens fibre nuclei	+++	"
ED 7	neural tube	+++	"
"	neural retina	+++	"
"	otic vesicle	+++	"
"	spinal ganglia	+++	"
"	Rathke's pouch	+++	"
ED 14	choroid plexus	+++	"
"	cerebral cortex:		"
"	pyramidal cells	+++	"
"	lens fibre nuclei	+++	"
"	equatorial cell nuclei	+++	"
"	cortical cell nuclei		"
"	nuclear cell nuclei		"
"	lens fibre cytoplasm	+	+++
ED 19	subtrochlear organ	+++	"
"	cerebellum:		"
"	Purkinje cells	+++	"
"	molecular layer	++	"
"	granular layer	++	"
"	white matter	+	"
"	gray matter	+	"

Abbreviations: ir, immunoreactivity; ND, not determined; -, no immunoreactivity; +++ = intense; + = weak staining

Fig 3.1. GH-immunoreactivity in neural tissues of the ED 3 embryo. A: Transverse section through the telencephalon (Te). The wall of the telencephalon is strongly staining for GH and the cells of this wall appear to have uniform intensity of staining density. The olfactory pit (Op) is also GH-immunoreactive and is of a similar staining intensity to the telencephalon. The amnion (a), mesoderm (M) surrounding the brain and epidermis (ep) of the head are also immunoreactive. T = teleocoel. Bar = 120υm. B: GH-immunoreactivity is abolished in the telencephalon, olfactory pit, mesoderm, and epidermis when the primary antibody is preabsorbed with recombinant cGH. C: The otic vesicle (Ot) and associated nerve ganglia show strong GH-immunoreactivity. IX = 9th cranial nerve (glossopharyngeal), VII & VIII = 7th and 8th cranial nerves which are closely associated and it is impossible to distinguish between them (facial/acoustic nerve) Bar = 60μm. D: Preabsorption of the αGH1 abolishes staining; this section shows the same area as C. E: The semilunar nerve ganglion or Vth cranial nerve is also strongly GH-immunoreactive. Bar = 60μm. F: The neural tube (Nt) towards the posterior of the embryo is similarly GH-immunoreactive as other areas of the brain. N = notochord. Bar = 60μm. Tissues were stained using the ABC technique and αGH1.

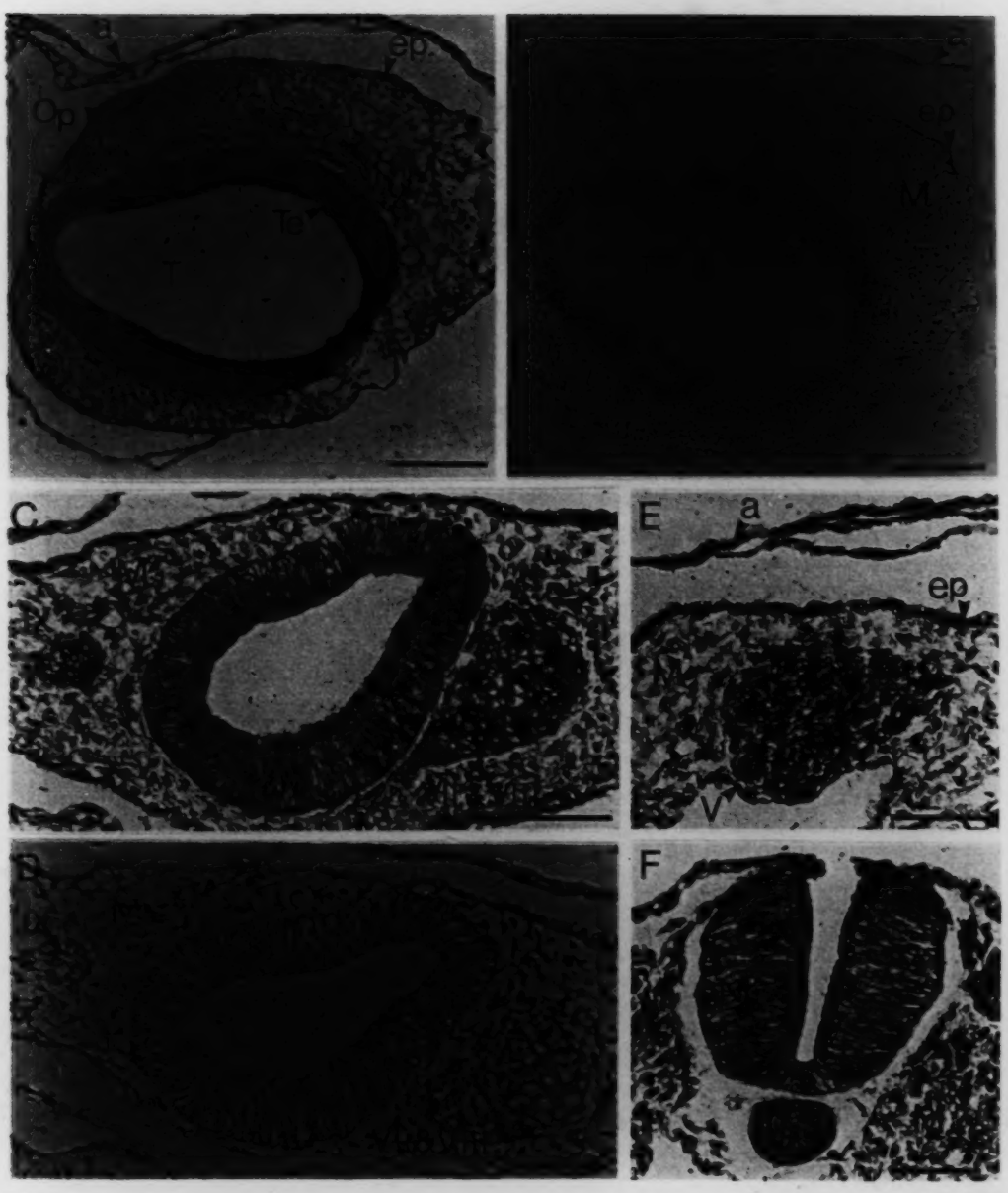

a
ep
Op
To
M
C
E
a
ep
V
F

Fig 3.2. GHR-immunoreactivity in neural tissues of the ED 3 embryo. A: GHR-immunoreactivity (transverse section) was observed in all major divisions of the brain and the mesencephalon (Me) is shown as a representation. B: GHR-immunoreactivity was observed in a similar distribution to GH-immunoreactivity. The otic vesicle (Ot) and associated nerves (VII = facial/acoustic ganglia; IX = glossopharyngeal nerve) were strongly immunoreactive. Also shown is the notochord (N), amnion (a), epidermis (ep) and mesoderm (M). C: GH-immunoreactivity in the optic vesicle (Op) and diencephalon (D) are GHR-immunoreactive. D: This section shows the sub-lunar ganglion is strongly GHR-immunoreactive. Also shown are the amnion (a), epidermis (ep) and mesoderm (M). PBS or NRS controls were used to replace the GHR antibody and did not result in staining (data not shown). Bar = 100μm. Tissues were stained using the ABC technique and αGHR as the primary antibody.

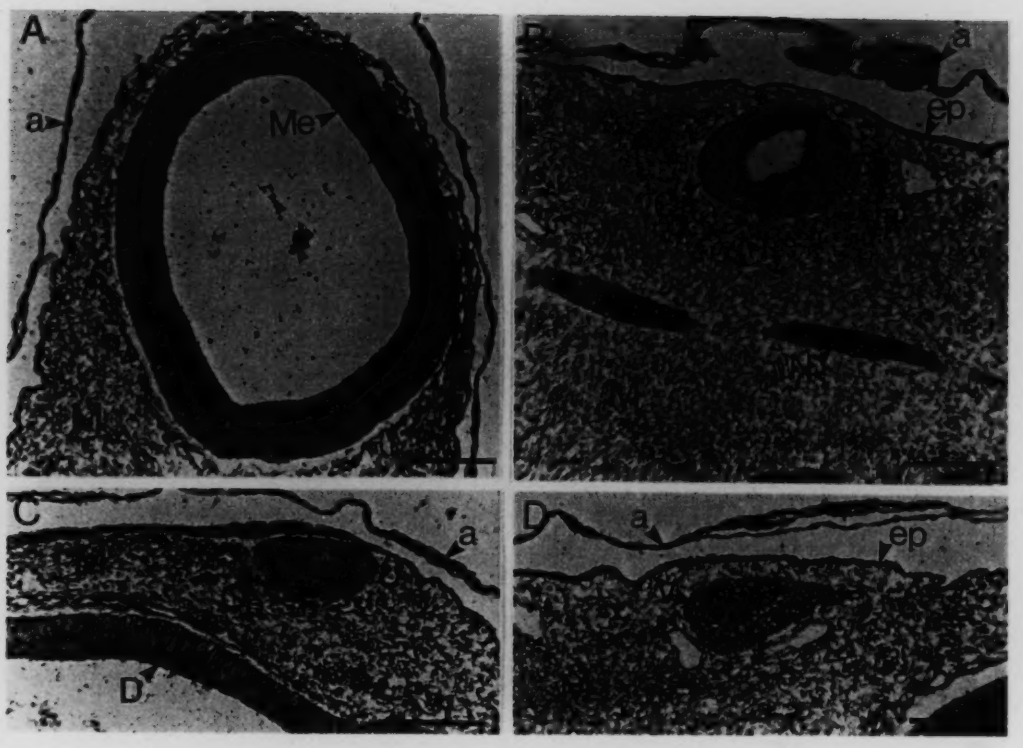

A
a
Me
a
ep
C
a
D
D
a
ep

Fig 3.3. GH-immunoreactivity in the eye of the ED 6 embryo. A: Transverse section of the optic cup. The neural retina (nr) is strongly GH-immunoreactive and the staining density was uniform throughout the structure. Staining was also seen in the surounding tissue behind the eye and in the inner layer of the amnion (a). It is also of interest that the outer layer of the amnion was not significantly stained. The lens (l) is also GH-immunoreactive and a high power magnification can be seen in C. B: Preabsorption of αGH1 with recombinant cGH completely abolished staining in a similar section to A. C: The cytoplasm of the lens fibre cells is less immunoreactive than the nuclei. Notice that some nuclei in the central lens fibre cells (C) are lightly staining or not immunoreactive. This is the region in which the nuclei of these cells are degenerating. The nuclei of the cortical lens fibre cells (Co), however, appear to be all immunoreactive. The epithelium (ep) of the lens is more densely stained than the cytoplasm of the lens fibre cells, and the nuclei in the cells of this layer are not distinguishable. The amnion (a) can also be seen outside the head region. A and B, Bar = 200μm, C, Bar = 50μm. Tissues were stained using the ABC technique and αGH1 as the primary antibody.

A
nr
l
ep
B

Fig 3.4. GH-immunoreactivity in the ED 7 eye. A: Transverse section through the head of a 7 day embryo showing part of the developing eye. The neural retina (nr) is very intensely stained but the mesoderm (m) which lies above the neural retina is much less immunoreactive, suggesting that staining is specific. The head epidermis, (ep) was also strongly immunoreactive. B: High power of the neural retina which lies below the pigmented retina (pr). Notice that scattered cells within the mesoderm was lightly stained (arrows). C: The immunoreactive staining of was completely abolished when the primary antibody was preincubated with recombinant cGH. Bar = 50μm. Staining was achieved using the ABC technique and αGH1 as the primary antibody.

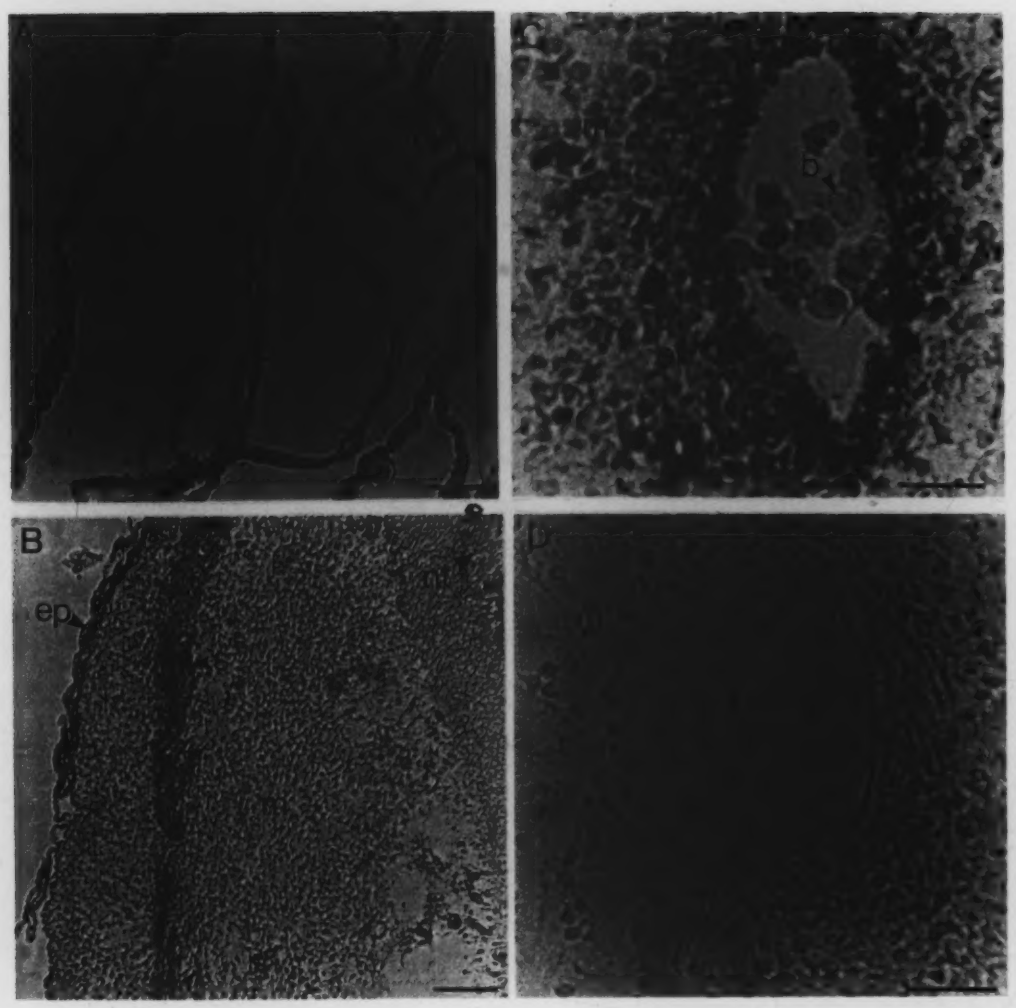

B
ep

Fig 3.5. GH-immunoreactivity in the ED 7 brain and nerve ganglia. GH-immunoreactivity was observed in the diencephalon (Di) wall (Fig 3.6A). Individual cells were visible in this wall and some appeared to be more intensely immunoreactive than others (arrows). Rathkes' pouch (Rp) was also very strongly stained with the αGH1. Scattered immunoreactive cells were also present in the head mesoderm (M). B: The otic vesicle was also very strongly immunoreactive (Ot). This structure will eventually develop into the ear. Several cranial nevre ganglia were also immunoreactive. The VIII (acoustic) ganglion, which is located to the side and below the otic vesicle was immunoreactive (Fig 3.6B). C: In discrete cells of the V or semilunar ganglion there was intense GH-immunoreactivity (Fig 3.5C). Preabsorption of the primary antisera with recombinant cGH or its replacement with NRS failed to result in any staining (data not shown). Bars = 40μm. Tissues were stained according to the APAAP technique with αGH1 as the primary antibody.

M
Rp
Di
D

M

M

Fig 3.6. GH- and GHR-immunoreactivity in the lens of the ED 14 embryo. A: GH-immunoreactivity is particularly localised to the nuclei of the lens fibre cells (n) from the cortical region (Co). There appears to be a larger proportion of cells in the central region (C), in which the nuclei are lightly stained. B: Preabsorption of the αGH1 abolished staining. C: GHR-immunoreactivity was seen throughout the lens and was not localised to the nuclei (n). The cytoplasm of all lens fibre cells was densely immunoreactive for GHR. Bar = 50μm. Tissues were stained according to the ABC technique using αGH1 as the primary antibody.

A
ep
ep

Fig 3.7. GH-immunoreactivity in the choroid plexus and the cerebral cortex of the ED 14 chick. A: The choroid plexus (Ch) is strongly GH-immunoreactive. B: Staining is abolished with preabsorption of the primary antibody with recombinant cGH. C: Various layers of the gray matter of the cerebral cortex were GH-immunoreactive. At this stage the cortex is not fully differentiated although layers typified by cell type were distinguishable. The molecular layer (Ml) is characterised by fewer cells and fibres. The pyramidal layer (Pl) is characterised by large pyramidal neurons. The bar represents layers which consisted of pyramidal neurons of small-intermediate size and neuroglial cells. D: Preabsorption of αGH1 with recombinant cGH completely abolished staining in a similar section to C. Bar = 50μm. Tissues were stained according to the ABC technique and αGH1 as the primary antibody.

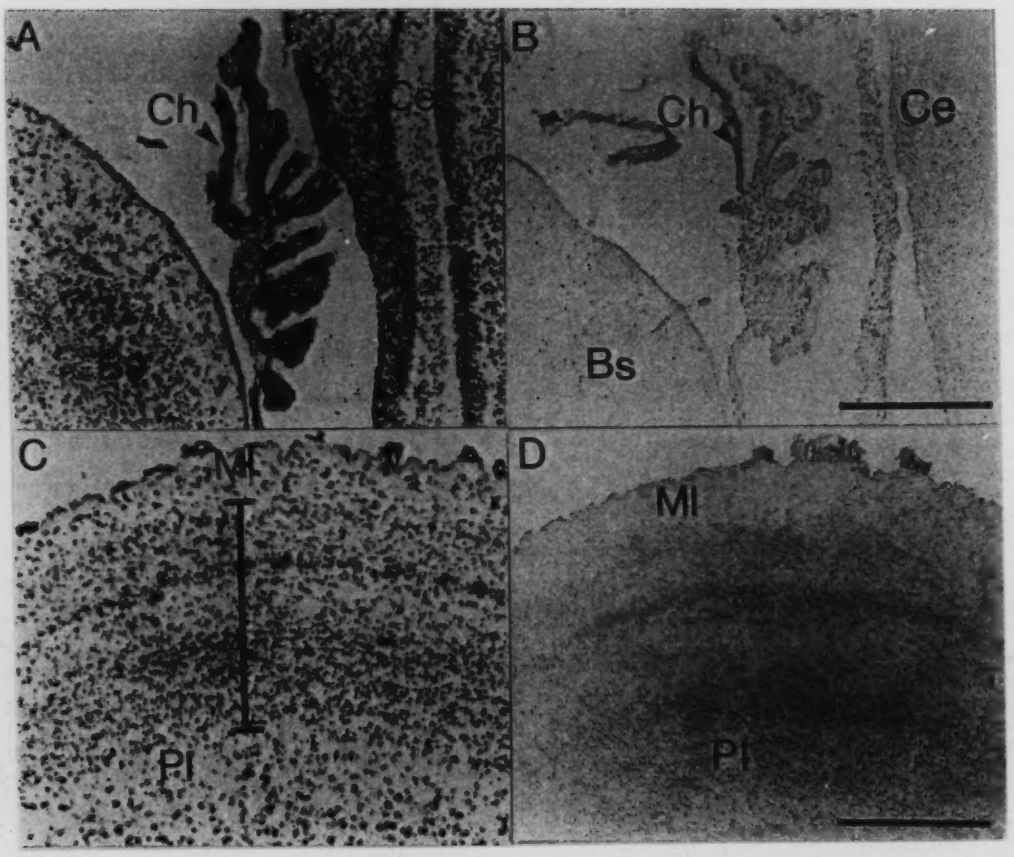

A
Ch
Ce
B
Ch
Ce
Bs
C
Ml
Pl
D
Ml
Pl

Fig 3.8. GH-immunoreactivity in the ED 14 chick cerebral cortex. A: Neuroglial cells from the gray matter of the cerebral cortex (probably astrocytes). B: Small and densely spaced cells from the white matter of cerebral cortex are seen to be GH-immunoreactive. The white matter is characterised by a higher density of neuroglial cells. C: Cell bodies of large pyramidal neurons (P) surrounded by various neuroglial cells (N). These pyramidal neurons send their axons towards the white matter and in this section the perikaryon is seen to be GH-immunoreactive. The nucleus (n) of these cells is strongly GH-immunoreactive. D: Immunoreactive cells from the pyramidal cell layer showing dense nuclear (n) staining and less dense cytoplasmic (c) staining. E: Nerve tract fibres from the white matter of the cerebral cortex (N) are also GH-immunoreactive. F: An similar area to A shows that preabsorption of the αGH1 with recombinant cGH completely abolished staining. Bar = 10μm. Staining was achieved using the ABC technique and αGH1 as the primary antibody.

hν

Fig 3.9. GH-immunoreactivity in the cerebellum of the ED 19 embryo. A: Low power section of the layers of the cerebellum stained with αGH1. The molecular layer (Ml) is not immunoreactive. The gray (Gr) and white matter (Wm) are immunoreactive. Bar = 50μm. B: At the junction between the molecular and granular layers are the large Purkinje cells (P). These are densely immunoreactive in their perikarya, although their axons which penetrate into the gray matter are not immunoreactive. The dendrites which branch into the molecular layer are also not immunoreactive. C: Preabsorption of the αGH1 with recombinant cGH completely abolished staining in the cerebellum. Bar = 10μm. Staining was obtained using the ABC technique and αGH1 as the primary antibody.

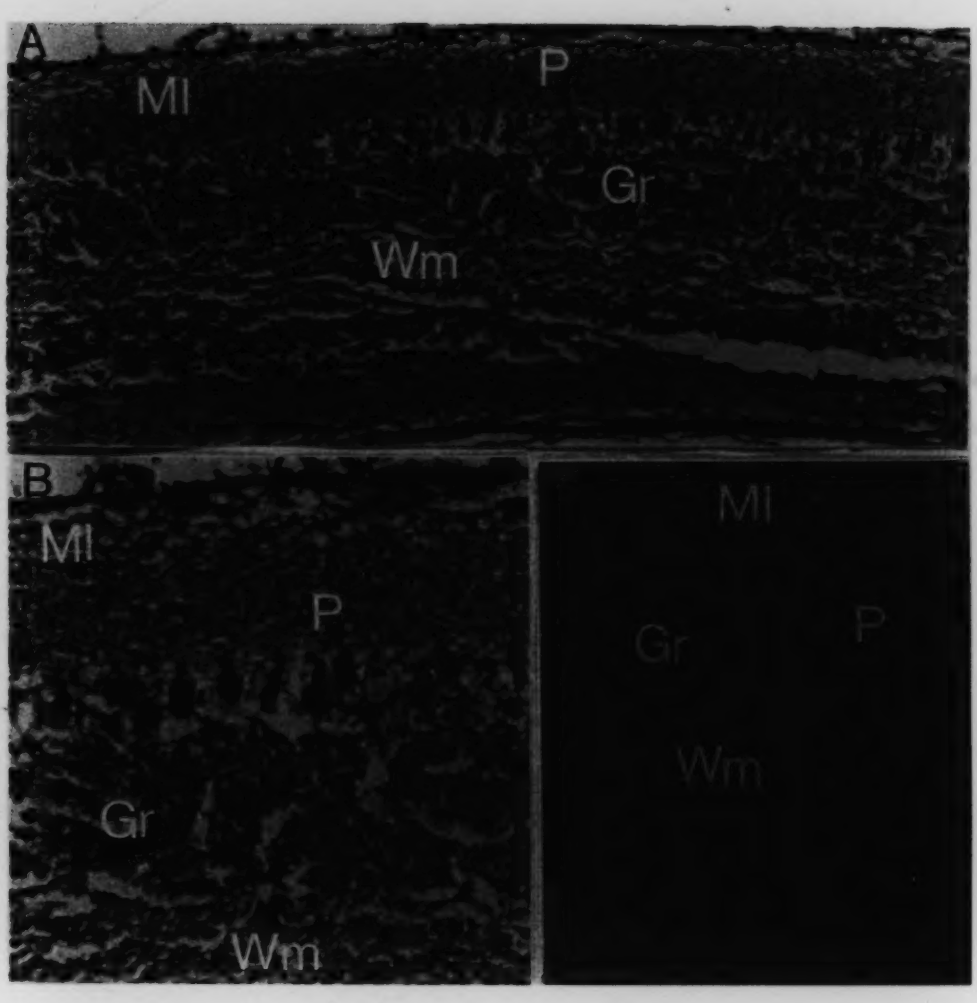
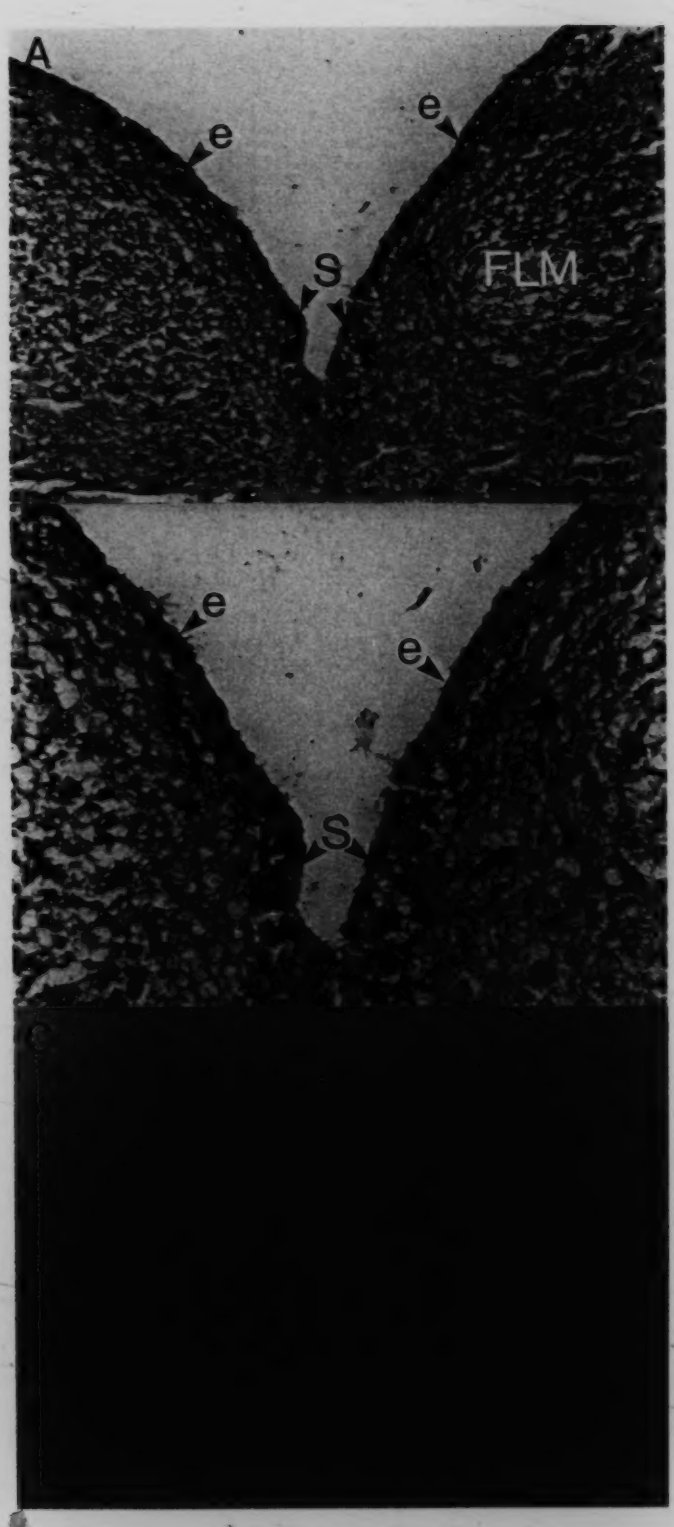

A
Ml
P
Gr
Wm
B
Ml
P
Gr
Wm
Ml
Gr
P
Wm

Fig 3.10. GH-immunoreactivity in the subtrochlear organ of the ED 19 embryo. A: The subtrochlear organ (S) strongly stained for GH. The subtrochlear consists of modified ependymal cells and is continuous with the ependymal layer (ep) lining the ventricle, that were also GH-immunoreactive. GH-immunoreactivity is also seen in the developing fasiculus longitudinalis medialis (FLM) nerve tract. B: High power magnification of A. C: Preincubation of the primary antibody with recombinat cGH resulted in complete loss of staining. Bar = 50μm. Staining was achieved using the ABC technique and αGH1as the primary antibody.


A
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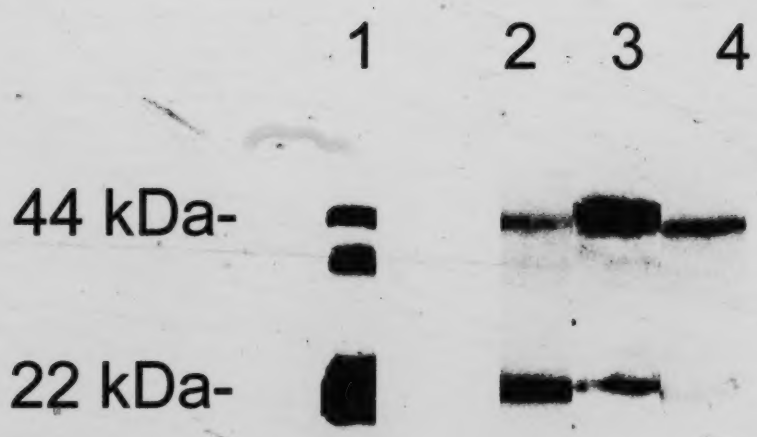

Fig 3.11. Western analysis of GH-like proteins in neural tissues of the ED 7 embryo. Homogenates of adult pituitary (lane 1), eye (lane 2), brain (incuding pituitary) (lane 3) and brain excluding pituitary (lane 4) were subjected to Western blotting. GH-like proteins of appropriate size (22kDa) are present in the ED 7 eye and brain. The larger 44kDa GH-like proteins probably corresponds to GH dimers. Protein homogenates were obtained from a pool of at least 5 embryos.

Fig 3.12. Analysis GH transcripts by the polymerase chain reaction (PCR) in the head of ED 7 and 9 embryos . Total RNA was obtained from the head of ED 7 (7h) and ED 9 (9h) embryos and reverse-transcribed. Moieties were amplified by 30 cycles of (PCR) in the presence of oligonucleotide primers (GH_3 and GH_5) for chicken cDNA. RNA was obtained from a pool of at least 5 embryos.

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Chapter 4

General Discussion

I. Overview and Summary

Growth hormone is the primary hormone responsible for regulating postnatal growth, but its role in the development and growth of vertebrate embryos is uncertain (Browne and Thorburn, 1989). Pituitary GH does not appear to be involved during the embryonic/foetal period for several reasons. Firstly, the pituitary gland does not contain functional GH-secreting cells until the last third of embryogenesis in avians (Porter, 1997) and towards the end of gestation in rodents (Khorram *et al.* 1983; Hemming *et al.* 1986). Secondly, pituitary ablation or decapitation does not result in retardation of total body growth in early avian (Thommes *et al.* 1987) and mammalian embryos (McCusker and Campion, 1990). Thirdly, exogenous GH administration has failed to increase the total body growth of embryos in many studies (Thommes *et al.* 1992).

However, extra-pituitary GH and GH receptor (GHR) have been detected in embryonic and foetal tissues and GH may, therefore, regulate the growth and differentiation of embyronic or foetal tissues rather than the "whole body". Indeed, exogenous administration of GH has significant effects upon restoring tibial length in decapitated chick embyros (Thommes *et al.* 1992), on stimulating the proliferation of foetal mouse osteoblasts (Slootweg *et al.* 1988) and in inducing metabolic actions in a number of foetal tissues (Stracke *et al.* 1984; Strain *et al.* 1987). Therefore, extra-pituitary GH could account for the growth of the embryo or foetus in a period of pituitary GH deficiency.

Based upon this hypothesis, this thesis investigated the expression of GH and GHR expression in the chick embryo.

In Chapter 2 the distribution and ontogenic pattern of extra-pituitary GH expression was examined. There are recent studies which have detected extra-pituitary GH in foetal mice (Panteleon *et al.* 1997), rats (Hojvat *et al.* 1992), and chick embryos (Wang, 1989) but, this is the first study to show the expression of GH and GHR during organogensis in tissues of the early chick embryo (ED 3-8). A study by Wang (1989) also found GH-immunoreactivity in early chick embryos, but he only investigated the embryonic discs of ED 1 and 2 embryos and the reproductive tracts of ED 7, 10, 12 and 13 embyros. Moreover, the specificity of his immunocytochemical staining was not determined and his data is unconvincing. Although Wang showed cross-reactivity of the extra-embryonic membrane to a porcine GH antibody, only the outside of the membrane was immunoreactive and other tissues were devoid of GH staining. His results therefore contrast with my finding of ubiquitous GH-immunoreactivity in early embryos (Chapter 2) and its concentration in the innermost layer of the extra-embryonic membrane. Since peripheral cells of tissue sections are often non-specifically labelled by many antisera, the specificity of Wangs (1989) is in doubt.

In Chapter 3 the ontogenesis of extra-pituitary GH expression in neural tissues of the chick embryo was investigated. Previous studies have detected GH expression in prenatal neural tissues of rodents (Hojvat *et al.* 1992), and although it is known

that GH is expressed in postnatal chicken brain (Render *et al.* 1995b), this was the first study to investigate avian embryonic neural tissues for the presence of GH. Furthermore, this study demonstrated that GHR is also expressed in neural tissues during early development.

There is considerable evidence postulating GH as a differentiation factor in prenatal life. A recent study (Zhang *et al.* 1997) detected immunoreactive GH, GH binding protein and GHR in rat embryonic odontogenic tissues, before the foetal pituitary becomes competent (Rieutort, 1974). Immunoreactivity of the GHR increased in several cell types as they began to differentiate, suggesting that GH is influencing . It is well established that exogenous GH promotes the differentiation of several cell types, although the precise mechanisms are not clear.

Growth hormone regulates postnatal growth, primarily by inducing the production of hepatic insulin-like growth factor-I, which then enters the bloodstream and acts in an endocrine way to induce the proliferation and growth of the target cells. IGF-I has also been shown to be important in prenatal growth and development and extra-pituitary GH could therefore, regulate embryogenesis by inducing extra-hepatic IGF-I production and through local autocrine or paracrine actions of IGF-I produced in developing tissues. Indeed, GH has been shown to stimulate the expression of IGF-I in foetal mouse chondrocytes and thereby promote their differentiation (Slootweg *et al.* 1988).

II. Mechanisms of extra-pituitary GH action

An important question arising from the results of this thesis is how extra-pituitary GH within embryonic tissues influences growth and development. It is known that the GHR is expressed in many extra-pituitary tissues, such as neural (Hull and Harvey, 1997b), immune (Hull *et al.* 1996), and skeletal (Monsonego *et al.* 1993) tissues and exogenous GH autoregulates GHR's in these sites (Monsonego *et al.* 1993; Hull and Harvey, 1997b). Furthermore, since GHR's also appear to be ubiquitously expressed in embryonic and foetal tissues, extra-pituitary GH could be acting in paracrine or autocrine ways. This is supported by the recent demonstration of functional GHR and GH expression in preimplantation murine embryos (Panteleon *et al.* 1997). Indeed, a role for GH in these embryos was demonstrated by the ability of exogenous GH to induce glucose uptake and protein synthesis.

III. Evolutionary Implications

The expression of GH in extra-pituitary tissues before the expression or release of pituitary GH raises intriguing questions pertaining to the origins, ancestral roles, and regulation of GH.

Both GH and closely related prolactin (PRL) have been isolated from examples of all vertebrates (mammalia; aves; reptilia; amphibia; pisces; elasmobranchii). GH has also been detected in the lamprey (Wright, 1986) which is a member of the Division Agnatha, which itself diverged from the rest of the

vertebrates (Division Gnathostomata) approximately 500 million years ago. This would also suggest that endocrine mechanisms evolved after paracrine mechanisms. This logical deduction is often overlooked today and the paracrine and autocrine action of hormones are still, largely, unexplored. It is possible, therefore, to envisage GH's ancient role as a local growth factor which later came to be predominantly expressed within the pituitary gland as higher animals evolved and homeostatic mechanisms became essential to the functioning of these organisms. The almost universal phylogenetic expression of GH would suggest that its roles were of fundamental importance and it is likely that those roles are retained in present day animals.

Due to its name, GH is often thought only to be a growth-promoting hormone but its plethora of biological actions suggests that GH is a "biological-jack-of-all-trades". Indeed, GH has effects upon protein, carbohydrate and lipid metabolism, osmoregulation, reproduction, adrenal steroidogenesis, cellular proliferation and differentiation, neurotransmission, central behaviour, and immune function (see Harvey *et al.* 1995). Therefore, the ancestral role of GH is far from clear. However, exogenous administration of mammalian GH has GH-promoting actions in molluscs and arthropods. This activity suggests GH stimulated growth in the deuterostomes and protostomes and that its growth-promoting activity is at least 500 million years old.

IV. Regulation of GH

The expression of GH was thought to be restricted to the anterior pitiuitary somatotrophs by the pituitary specific transcription factor (PIT-1) (Theill and Karin, 1993). Indeed, GH-expression in somatotrophs (day 16 in chickens) closely follows PIT-1 expression in these cells (Porter *et al.* 1997). However, the ectopic transcription of the GH gene in chick embryonic tissues could suggest that PIT-1 is expressed in extra-pituitary tissues, or that the expression of extra-pituitray GH is independent of PIT-1. PIT-1 has also been detected in haematopoetic tissues (Harvey and Hull, 1997) which express GH suggesting that this transcription factor is not restricted to the pituitary.

Further studies could be undertaken to investigate the possibility that the differentiation of GH-secreting cells in extra-pituitary tissues of the early embryo, is regulated by PIT-1. This could be achieved by using a probe to PIT-1 to ascertain whether PIT-1 was expressed within these embryonic tissues.

V. Future Studies

This thesis has clearly established that extra-pituitary GH is expressed within the tissues of the early chicken embryo. These results have raised a number of questions related to the origin and role of GH in embryonic development. These questions could be addressed in future studies that should also delineate the ontogeny and extinction of GH expression in discrete organs. For example, this thesis detected

GH-like immunoreactivity in the subtrochlear organ of the ED 14 embryo. This structure is postulated to be involved in the flexing and gross anatomical changes in the developing brain during a narrow time period during embryogenesis. It is therefore relevant to ask whether GH is expressed during a narrow time period when these changes are occurring, or whether its expression within this organ is during a wider period of embryogenesis.

This study has provided no evidence that GH is being secreted from cells within embryonic tissues. The reverse hemolytic plaque assay is a method which allows the detection of hormone secretion from individual cells (Neill and Frawley, 1983) and has been used to detect GH secretion from pituitary cells during chick embryogenesis (Porter, 1997). Therefore, the method could be employed to detect GH secretion from cells from certain embryonic tissues. If it is secreted this would support the possibility that it could have autocrine/paracrine roles. If it is not secreted, this would suggest GH has intracrine roles or has no role in embryogenesis. It is possible that the GH-immunoreactivity seen within embryonic cells in this study is due to sequestration from the blood. This would involve transport in the circulatory system from a production site or sites and this is possible as the system is functional at ED 2 of chick embryonic incubation (Romanoff, 1960). The possibility that GH is passing across the blood-brain-barrier could be investigated using radiolabelled GH.

Electron microscopy could have been used to investigate the sites of GH-immunoreactivity. This method would be able to detect GH on the plasma membrane

or within the intracellular component. If GH was found inside the endoplasmic reticulum and Golgi apparatus would suggest synthesis by the cell.

Functional studies are therefore also needed to determine if GH has roles during embryonic growth and development. This would involve investigations into the mechanisms of GH action, ands its intracellular site of action. This could be achieved by determining tissue responsiveness in terms of GH inducible genes (Burnside *et al.* 1997), to exogenous GH and in tissues in which GH or GHR gene expression has been blocked using sense oligonucleotides or in tissues in which GH or the GHR have been blocked by passibe immunoneutralisation. Such studies may identify genes such as GHRG 1 and II (Burnside *et al.* 1997) or IGF-I (Goddard *et al.* 1988) that respond to exogenous or endogenous GH during embryonic development. Similar *in vitro* or *in vivo* studies may also show that the proliferation and differentiation of cell- and tissue-types is sensitive to exogenous or endogenous GH. However, since many factors are likely to regulate the transcriptional activity of growth and differentiation factors, GH or GHR knockout may not, by itself, impair embryonic development. Indded, this redundancy may account for the almost normal embryonic development that occurs in dwarf chicken strains that are GH resistant as a result of a mutation in their GHR gene. In humans, Laron-type dwarfs similarly have a normal birth weight despite having a GHR defect and tissue GH resistance (Laron, 1984).

A method which is gaining popularity in developmental biology is the construction of replication-defective retroviruses which carry an inserted gene of interest (Tickle, 1992). The viral particles can be injected into certain tissues of an embryo and will spread locally as development proceeds. A dominant mutant GH receptor gene which results in a non-functional receptor would allow insight into the possible effects of GH during embyrogenesis. This method allows only certain tissues to be infected with the defective gene and therefore the effects of a particular protein upon a certain tissue can be studied.

Lens, chondrocyte and nerve cell cultures are all possible *in vitro* systems which are widely used to study the effects of growth factors upon certain developmental processes. These systems could be used to investigate the role of GH upon proliferation and differentiation. The developing lens is an ideal model to study cellular and molecular events during embryogenesis for several reasons (see Wride, 1996 for review). Firstly, lens cultures are relatively easy to set up and maintain. The system is very well understood and lens differentiation occurs in an orderly sequence. Furthermore, specific events such as proliferation can be measured by detecting the incorporation of certain substances into proliferating cells. These substances can be detected by immunocytochemistry. For example, lens cell nuclei undergo degeneration so that the lens is made transparent and light can pass through easily. Degenerating nuclei can be detcted by a technique known as terminal deoxynucleotide transferase mediated dUTP-biotin nick-end labelling (TUNEL). This

study detected GH-immunoreactivity within lens cell nuclei and it is possible to envisage a role for growth hormone in nuclear degeneration. It is known that certain genes (e.g. homeobox genes) are expressed during lens development and this knowledge could provide a basis for studying the possible gene expression effects of GH during lens development.

VI. Limitations of the study

The methodology used in the present study has a number of limitations. Although immunocytochemistry is a widely used technique which shows the distribution of immunoreactive substances, it does not indicate how much substance is present. The presence of immunoreactivity and adequate controls suggests that a particular substance is present within a tissue or cell but the immunoreactivity could be due to non-specific staining. The use of a panel of three antibodies in this study however, strongly suggests that the immunoreactivity is due to the presence of GH. Immunoreactivity achieved with the use of a monoclonal antibody which recognises a single epitope on the GH molecule, also supports the presence of GH. The high titre of these antibodies and the dilution of their use (1: 4000) also suggests they are unlikely to non-specifically react with proteins other than GH. Furthermore, Harvey and Scanes (1977), Porter (1995) and Berghman (1987) established by cross-reactivity studies that these antibodies were specific for GH and that they did not

cross-react significantly with any other hormone, even with closely related proteins like prolactin.

One antibody (αGH1) was used in the Western analysis of chick embryonic tissues. This data could be strengthened by using all of the antibodies in Western analyses and if GH-like proteins of appropriate size were detected by all 3 antibodies then the data would be more convincing. Further information could have been obtained from Western analysis by quantitating the amount of GH present per gram of protein. This data could have been compared to the amount of GH per gram of protein present in the pituitary gland.

The polymerase chain reaction (PCR) results in the selective amplification of a chosen region of a DNA molecule from the appropriate mRNA sequence and is a widely used technique today. This valuble procedure is extremely sensitive, detecting very small amounts of mRNA. This method is also reliable because the primers which are used to detect the ends of the DNA sequence of interest are complementary to these sequences and are unlikely to recognise any other sequences. PCR could have been extended to include DNA sequencing which would have confirmed that the gene was the GH gene.

VII. Summary

This preliminary study has detected GH-like proteins by immunocytochemistry using a panel of 3 antibodies. Western analysis revealed that

GH-like proteins of identical molecular size to pituitary GH are present in some embryonic tissues and PCR detected a mRNA sequence also identical to pituitary GH mRNA. Taken together, these results strongly suggest that GH-like proteins are present in chick embryonic tissues and raises questions regarding sites of GH synthesis in chick embryos. The detection of GHR-like proteins within certain tissues would suggest GH may posses biological activity in embryogenesis.

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